PHD

Effect of ischaemia on the activities of lipid metabolizing enzymes in perfused hearts from normal and diabetic rats

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Dedicated to my mother, father and sister.

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2. METHODS

Summary

This investigation was undertaken to study the mechanism of the effects of adrenergic and ischaemic challenge on the activities of lipid metabolising enzymes in perfused rat hearts. The activation state of phosphorylase was measured for comparison with the activities of triglyceride lipase (TGL) and glycerol 3-phosphate acyltransferase (GPAT).

Perfusion with adrenaline increased TGL activity and decreased GPAT activity. This inhibition of GPAT could be reversed on incubation of heart homogenates under dephosphorylation conditions and mimicked by incubation with cAMP-dependent protein kinase. Adrenaline inhibited microsomal GPAT activity only; the mitochondrial activity was unchanged. Homogenization of hearts in NaF enhanced the adrenaline-induced depression of microsomal GPAT activity.

Both perfusion of hearts and incubation of heart extracts with the protein kinase C activator 12-O-tetradecanoyl phorbol 13-acetate (TPA) inhibited microsomal GPAT activity; TGL activity was unaffected. Incubation of heart extracts under dephosphorylation conditions reversed the TPA-induced decrease in GPAT activity. Perfusion with TPA caused an activation of phosphorylase.

Perfusion with adrenaline and TPA produced identical changes in enzyme activity to those seen with TPA alone.

Induction of ischaemia activated TGL and inhibited GPAT activity (in homogenate or microsomal fraction). This effect has been attributed to cAMP-dependent phosphorylation. On reperfusion, TGL activity returned to control values but GPAT activity was further depressed. Phosphorylase activity increased during ischaemia and, on reperfusion, a further increase occurred. These changes are the mirror image of the changes in GPAT activity.

Preperfusion of hearts with Ca^{2+}-antagonists prevented the reperfusion, but not ischaemia, induced inhibition of GPAT.

Induction of ischaemia in isolated hearts from diabetic rats produced similar changes in enzyme activity to those occurring in normal hearts. However, there was no further inhibition of GPAT or activation of phosphorylase on reperfusion of diabetic hearts. Since, in normal hearts, Ca^{2+} antagonists prevented the reperfusion-induced fall in GPAT activity, this suggests that Ca^{2+} uptake during reperfusion is attenuated in diabetic hearts.

AIMS

The object of the work performed in this thesis was to investigate mechanisms involved in regulating the activities of lipid metabolizing enzymes (GPAT, TGL and CAT) during ischaemia and reperfusion of isolated perfused hearts from normal and diabetic rats.

(1) Heathers (1985) found evidence for an α_1-adrenergic activity upon reperfusion of previously ischaemic hearts which caused a decrease in GPAT activity without affecting TGL activity. Here, the mechanism of this α_1-effect was investigated by use of Ca^{2+} antagonists and phorbol esters (activators of PKC): two possible mediators of α_1-action are an increase in $[Ca^{2+}]_i$ and activation of PKC.

(2) Hearts from diabetic animals are known to have elevated levels of triglyceride. Therefore, control of enzymes of triglyceride metabolism may be altered in diabetes. Enzyme activities were also measured during ischaemia and reperfusion to determine whether similar changes occurred to those found in non-diabetic hearts.

Increases in the concentration of fatty acid metabolites have been associated with arrhythmias occurring during ischaemia; diabetic hearts have an elevated level of such metabolites and an increased incidence of ischaemia-induced arrhythmias. Knowledge of the control of enzymes involved in the generation and removal of fatty acid metabolites may help further our understanding of the mechanisms involved in arrhythmogenesis.

INTRODUCTION

1.1 Myocardial Lipid Metabolism

The heart is a constantly contracting muscular tissue and therefore a centre of intense metabolic activity. The substrates needed to provide energy for mechanical and electrical activity are supplied largely by the blood. However, the heart contains glycogen and lipid (triglyceride) stores which can be used when exogenous substrate supply is limited (for example during ischaemia) or when the heart is stimulated with catecholamines (when energy demand exceeds substrate supply from the blood).

a) Exogenous substrates

Fatty acids are utilized in preference to glucose; fatty acid oxidation comprising 60-70% of oxidative metabolism (Neely et al., 1972). Non-esterified (free) fatty acids depress glucose utilization by inhibiting pyruvate dehydrogenase activity, due to formation of acetyl-CoA units derived from fatty acid oxidation (Randle et al., 1970).

Fatty acids are available to the heart directly as albumin complexes in blood or as the products of lipoprotein lipase activity at the myocardial cell surface upon circulating chylomicra and very low density lipoproteins.

The heart also utilizes lactate, pyruvate, ketones and amino acids. These are not quantitatively major substrates but may become important under certain conditions e.g. in the normal heart during ischaemia and as ketone bodies in uncontrolled diabetic episodes.

b) Endogenous substrates

Triglyceride is the major energy store of the myocardium: substrate-free perfusion of the isolated rat heart results in depletion of glycogen stores within 5 mins. whereas contraction can be maintained by endogenous lipid for up to 45 mins. (Shipp et al., 1964). Catecholamines can stimulate endogenous lipolysis - this can be measured as increased rates of glycerol release (Christian et al., 1969; Severson et al., 1980) or a decreased tissue level of triglyceride (Crass et al., 1975). Exogenous glucose has little effect on endogenous triglyceride utilization but exogenous fatty acids inhibit endogenous lipolysis (Crass et al., 1975), as do elevated levels of endogenous fatty acids or long chain acyl CoA (Neely et al., 1972).

c) Intracellular metabolism

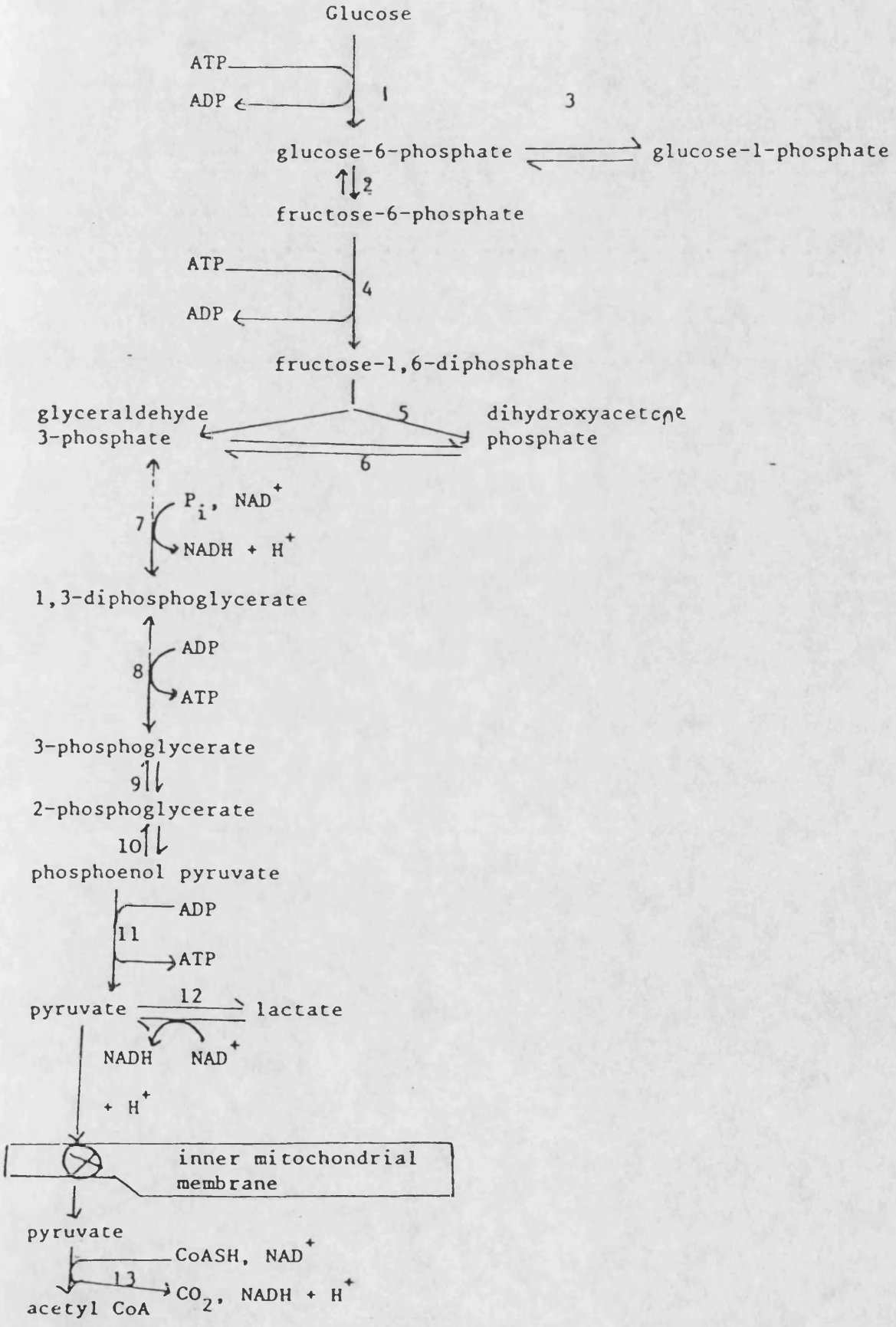
Myocardial metabolism is highly aerobic; mitochondria comprise approximately 30% of cardiac cell volume (Sobel, 1974). Energy generating pathways involve the normal routes of carbohydrate and lipid metabolism: (i) glucose is

Legend to Figure 1.1

1. Glucose kinase, hexokinase
2. Phosphoglucose isomerase
3. Phosphoglucomutase
4. Phosphofructokinase
5. Aldolase
6. Triosephosphate isomerase
7. Glyceraldehyde-3-phosphate dehydrogenase
8. Phosphoglycerate kinase
9. Phosphoglyceratemutase
10. Enolase
11. Pyruvate kinase
12. Lactic dehydrogenase
13. Pyruvate dehydrogenase

Figure 1.1

The glycolytic pathway



phosphorylated to glucose 6-phosphate which can either be stored as glycogen or enter glycolysis (see Figure 1.1) to produce pyruvate. The latter can enter the citric acid cycle (via pyruvate dehydrogenase activity) or be reduced to lactate (under conditions of reduced oxygen supply) (Neely and Morgan, 1974). (ii) fatty acids (from endogenous or exogenous sources) are activated to fatty acyl-CoA thioesters which enter mitochondria via the carnitine cycle (Figure 1.2). The resultant mitochondrial acyl CoA esters undergo β-oxidation (Figure 1.3) to produce acetyl CoA units which enter the citric acid cycle (Figure 1.4).

Lipid synthesis is also active in the myocardium acyl CoA esters react with glycerol 3-phosphate derived from glycolysis to form triglycerides (Denton and Randle, 1965) or can be used for synthesis of membrane phospholipids (Dawson, 1966).

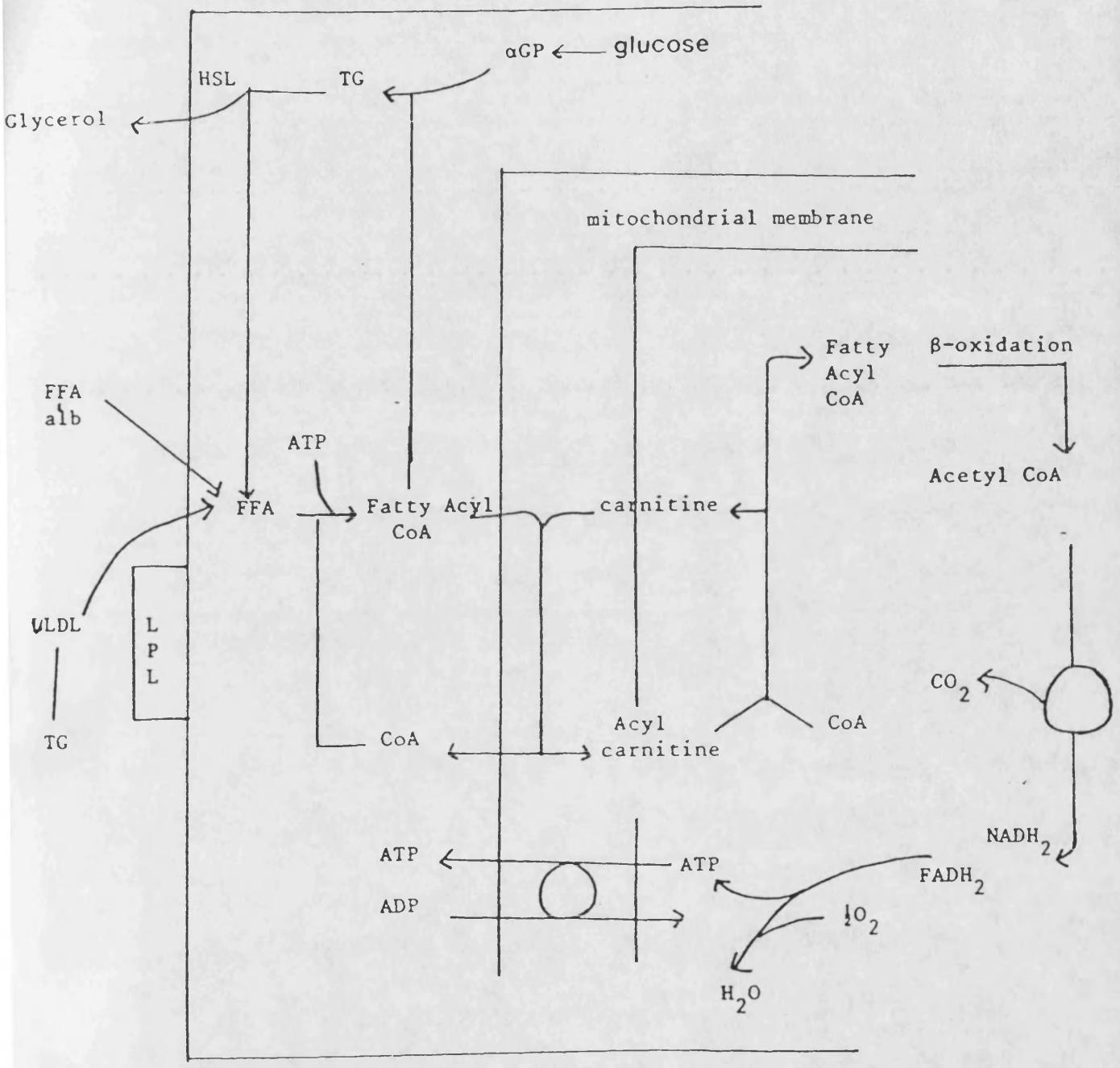
i) Lipases

Several discrete lipase activities have been described in the mammalian heart including lipoprotein lipase, mono- di- and tri-glyceride lipases and an acid lipase (Stam et al., 1986).

A neutral triglyceride lipase (TGL) (EC 3.1.1.3) controls the rate limiting step of endogenous triglyceride hydrolysis (Figure 1.5); hydrolysis is completed by the sequential action of diglyceride and monoglyceride lipases.

Figure 1.2

Lipid metabolism in cardiac muscle



From Severson (1979) Can. J. Physiol. Pharmacol.

Figure 1.3

β-oxidation of fatty acids

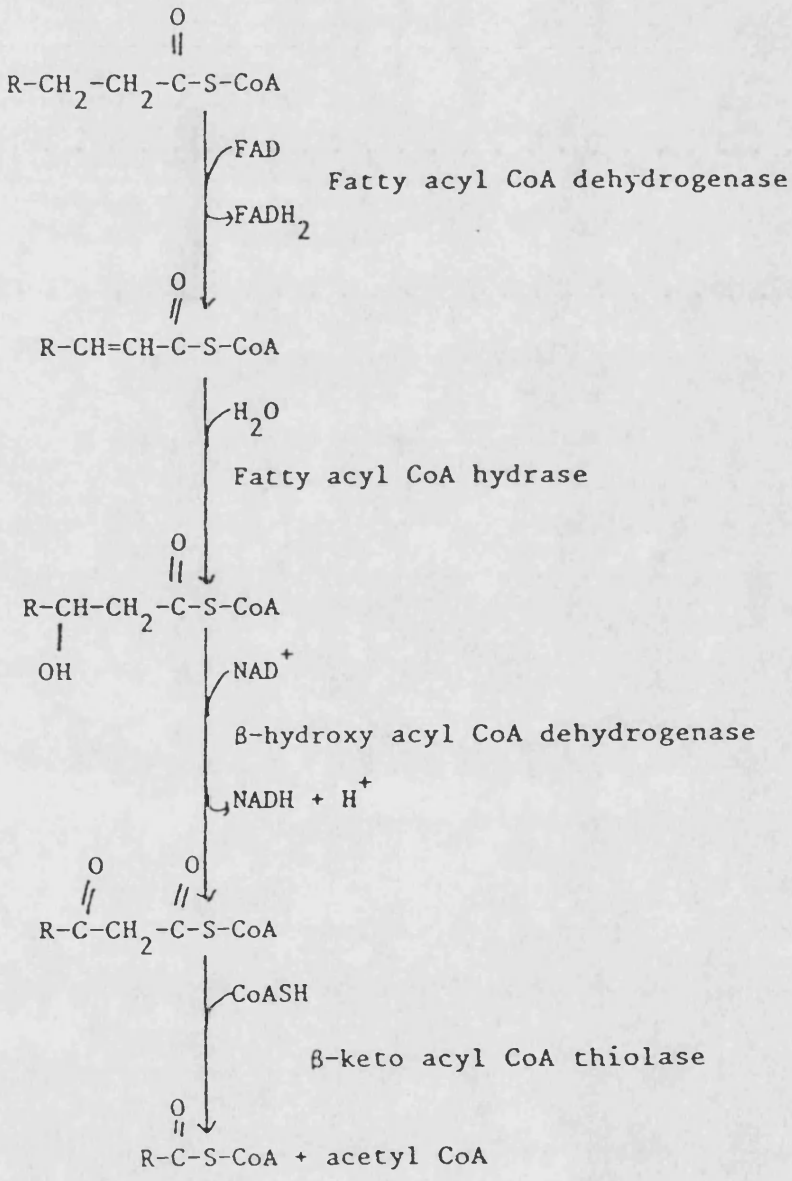
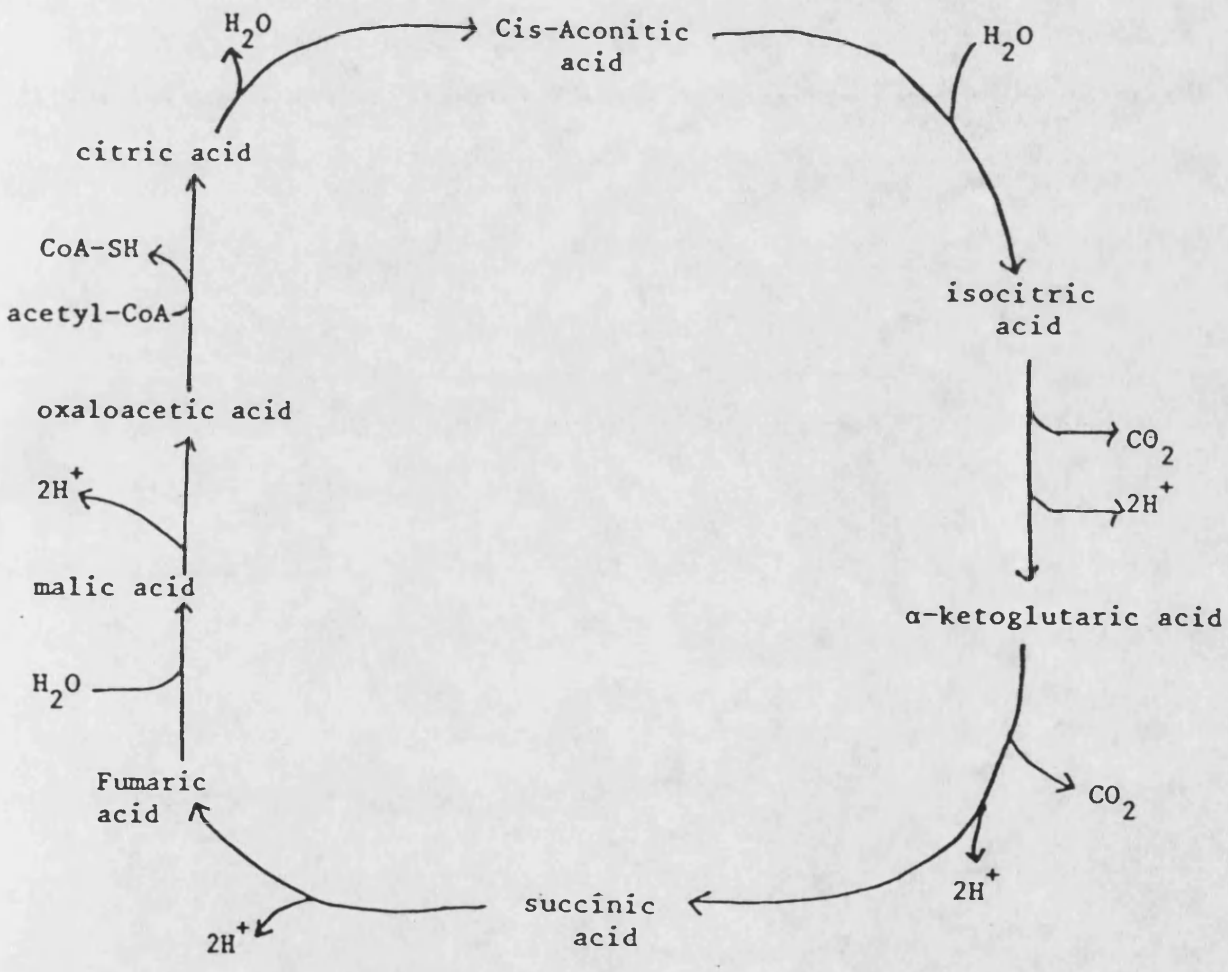


Figure 1.4

The citric acid cycle



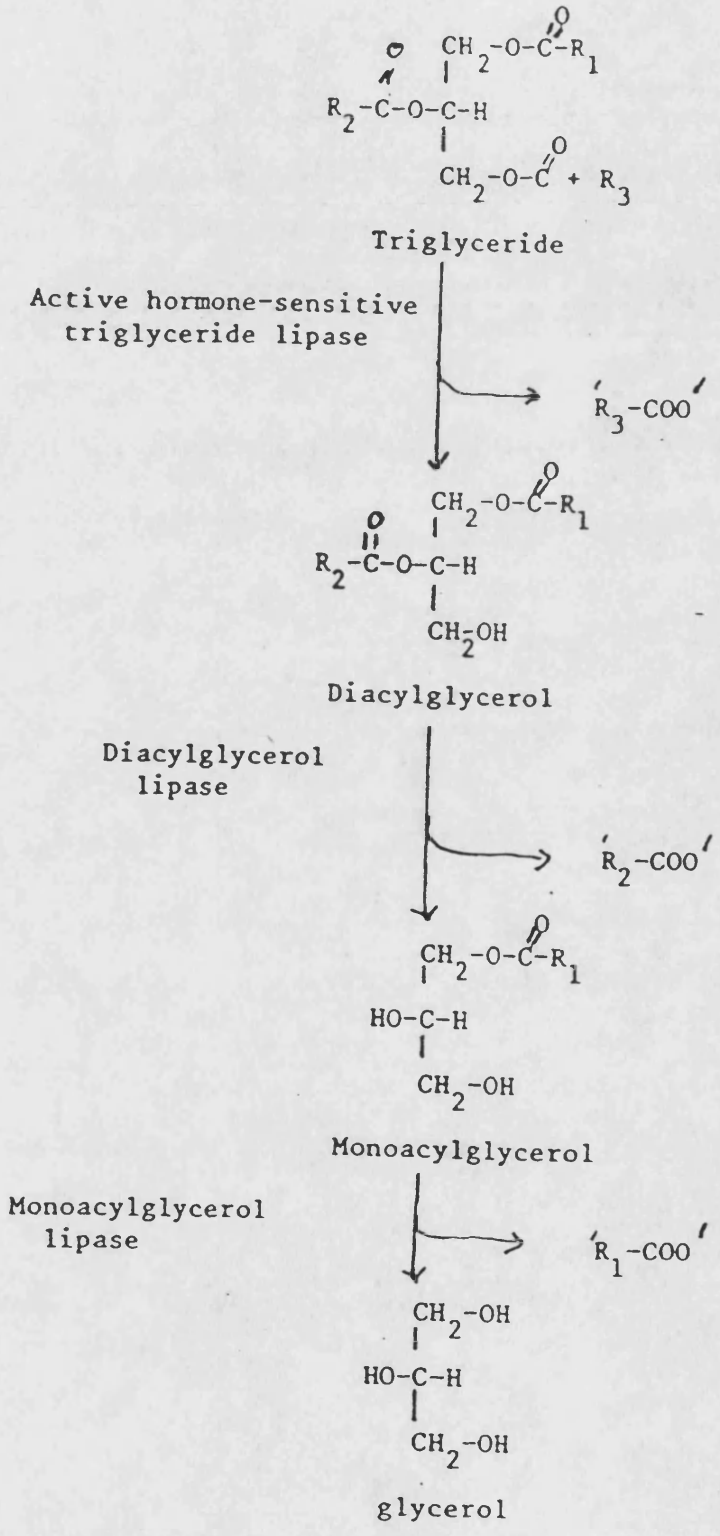
Hulsmann et al (1981), suggested that both acid and neutral lipases may contribute to endogenous lipolysis. However, since chloroquine (a lysosomotropic agent which inhibits the acid lipase) did not decrease glycerol output from perfused rat hearts (Rosen et al., 1981b; Severson et al., 1980), it can be concluded that it is the neutral TGL activity which essentially determines the rate of cardiac lipolysis.

The nature of this neutral lipase has been the subject of much controversy: in hearts perfused with heparin to remove vascular lipoprotein lipase (LPL), a residual LPL activity remains which could either be an intracellular storage pool or a soluble precursor (Hulsmann et al., 1982). However, several workers have identified a neutral lipase with properties distinct from those of LPL (Ramirez et al., 1985; Goldberg and Khoo, 1985). Thus, unlike LPL, TGL is not stimulated by heparin nor is it inhibited by NaCl and protamine sulphate. It is inhibited by NaF. Also unlike LPL, it is not activated by apolipoprotein CII.

In previous work from this laboratory, Al-Muhtaseb (1982) developed an assay whereby LPL and TGL could be distinguished. This was based on the assay pH, type of buffer and the presence or absence of serum (see "Methods" section for details).

Figure 1.5

Triglyceride hydrolysis



ii) Enzymes of triglyceride synthesis

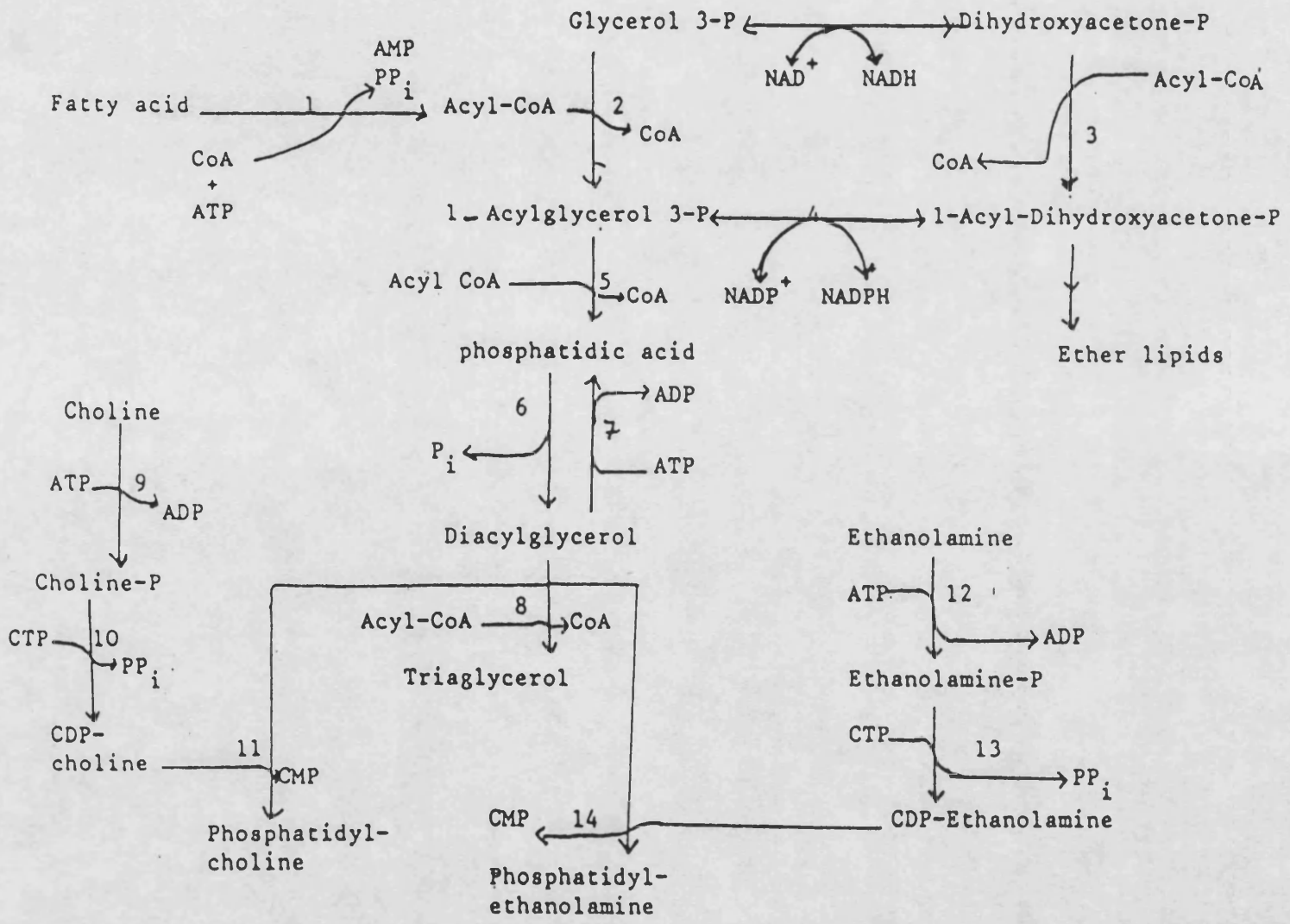
The pathway of mammalian triglyceride synthesis was first described by Kennedy et al. (1961). The overall pathway of triglyceride and phospholipid synthesis is shown in Figure 1.6. (review, Bell and Coleman, 1983). Fatty acids are activated to fatty acyl CoA thioesters by one of several fatty acyl CoA (FA-CoA) synthetases, depending on acyl chain length. Cytosolic FA-CoA esters can then undergo either esterification to form glycerolipids or mitochondrial oxidation. The initial, rate limiting step in triglyceride synthesis is catalysed by glycerol 3-phosphate acyl transferase (GPAT) (see next section). The reaction proceeds to 1,2-diacylglycerol phosphate (phosphatidate) by the action of 1-acyl glycerol 3-phosphate (lysophosphatidate) acyltransferone. The next enzyme, phosphatidic acid phosphatase (PPH) exists in an Mg^{2+}-dependent and an Mg^{2+}-independent form (depending on cellular location). Diacylglycerol acyltransferase catalyses the final step in triglyceride synthesis and is the only enzyme in the pathway unique to triglyceride synthesis.

Glycerol 3-phosphate acyl transferase (GPAT) (EC 2.3.1.15)

The reaction of FA-CoA with glycerol 3-phosphate takes place in two steps (Figure 1.7). The first step, catalysed by GPAT, involves esterification of a long chain fatty acyl CoA group into position 1 of glycerol 3-phosphate to form 1-acyl glycerol 3-phosphate (lysophosphatidate). The 2nd

Figure 1.6

Long chain fatty acid esterification (glycerolipid biosynthesis)

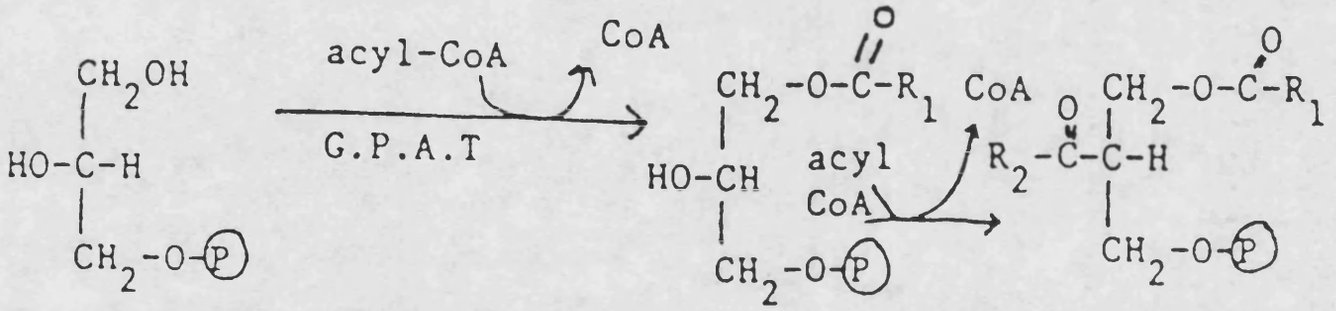


Legend to Figure 1.6

1. Fatty acid CoA ligase
2. Glycerol 3-phosphate acyltransferase
3. Dihydroxyacetone-P-acyltransferase
4. Acyl(alkyl)dihydroxyacetone-P-oxidoreductase
5. Lysophosphatidic acid acyltransferase
6. Phosphatidic acid phosphatase
7. Diacylglycerol kinase
8. Diacylglycerol acyltransferase
9. Choline kinase
10. Choline-P-cytidyltransferase
11. Diacylglycerol cholinephosphotransferase
12. Ethanolamine kinase
13. Ethanolamine-P-cytidyltransferase
14. Diacylglycerolethanolaminephosphotransferase

Figure 1.7

Fatty acid esterification



α-glycerophosphate

lysophosphatidic acid

1,2 Diacylglycerol phosphate (phosphatidate)

step involves esterification at the 2-position to form diacylglycerol 3-phosphate (phosphatidate). Both activities may reside in the same enzyme (Bell and Coleman, 1983).

GPAT activity has been found in both mitochondrial and microsomal fractions in rat heart, liver and adipose tissue (Daae and Bremer, 1970); the two subcellular locations contain different isoenzymes with different properties (Nimmo, 1979). In adipose tissue less than 10% of total GPAT activity is present in the mitochondrial fraction, but in liver and heart the activities are distributed approximately equally (Bremer et al., 1976). Most work outside this laboratory has focussed on the enzyme from adipose tissue: microsomal GPAT activity is inhibited by sulphydryl reagents such as N-ethyl maleimide whilst the mitochondrial activity is unaffected; this property can be used to distinguish between the two isoenzymes (Saggerson et al., 1979). The two isoenzymes also differ in their pH optima and Km's for palmitoyl CoA. The principle site of glycerolipid synthesis is the endoplasmic reticulum where GPAT activity is not specific, using palmitoyl CoA and oleyl CoA with equal efficiency (Halder, 1978). In contrast, the mitochondrial enzyme has a lower Km for palmitoyl CoA (Monroy et al.1973) and may be important for precise specificity during esterification.

d) Oxidation of fatty acyl CoA

Cytosolic fatty acyl CoA thioesters can either be esterified to form glycerolipids, as has been described, or oxidized within the mitochondrion (McGarry and Foster, 1980). In liver, the activity of the 1st enzyme in each pathway has a reciprocal relationship (Borrebaeks et al., 1974); during starvation, for example, the activity of GPAT fell and that of carnitine acyltransferase$_I$ (CAT_I) rose, the reverse occurring on refeeding. However, in heart the activities of GPAT and CAT_I were found not to alter with the nutritional state of the animal (Borrebaeks et al., 1974).

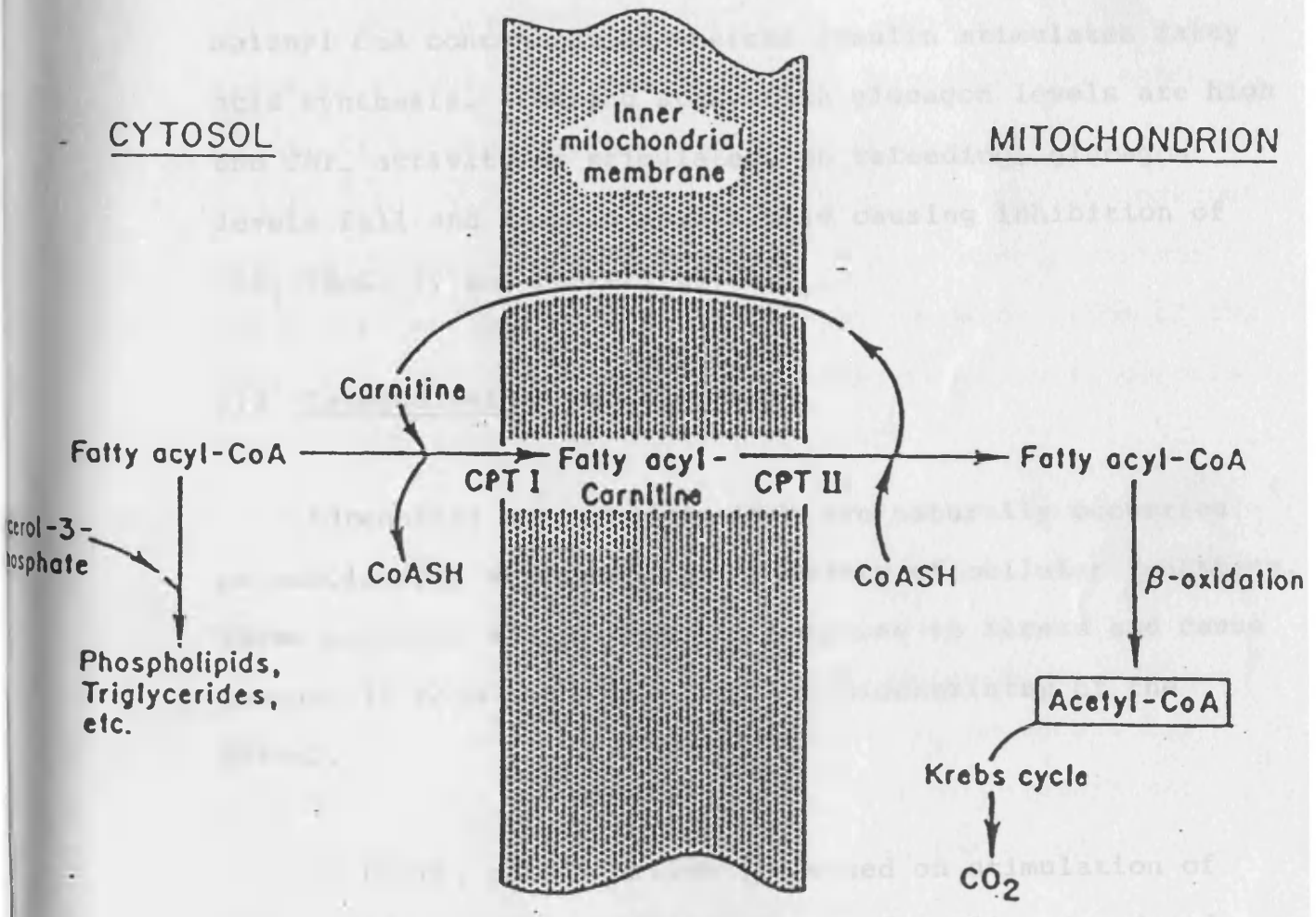
Carnitine Acyltransferase (CAT) (EC 2.3.1.21).

CAT catalyses the transfer of fatty acyl CoA esters from the cytosol into the mitochondria (Figure 1.8). It exists in two forms located on the outside (CAT_I) and inside (CAT_{II}) of the inner mitochondrial membrane (Bremmer, 1977). CAT_I catalyses the formation of acylcarnitine from acyl CoA and carnitine; acyl carnitine is then transferred to the inside of the inner mitochondrial membrane where CAT_{II} catalyses the reverse reaction to release acyl CoA into the mitochondrial matrix.

Malonyl CoA (an intermediate in fatty acid synthesis) is a potent inhibitor of CAT_I (but not CAT_{II}) in rat liver and heart (McGarry et al., 1978a,b,c; Saggerson, 1982). The mechanism of inhibition is not known, but may involve a

Figure 1.8

Transfer of fatty acyl CoA across the inner mitochondrial membrane



from McGarry and Foster, 1980.

competitive inhibition against acyl-CoA substrates. The concentration of malonyl CoA in both liver and heart fluctuates with the nutritional status of the animal (McGarry et al., 1977, 1983). Thus during starvation, fatty acid synthesis and malonyl CoA concentration are low whilst CAT_I activity and fatty acid oxidation are elevated (McGarry and Foster, 1980). These changes appear to be mediated by the glucagon/insulin ratio (McGarry et al 1978b): glucagon inhibits fatty acid synthesis and thus decreases malonyl CoA concentration whereas insulin stimulates fatty acid synthesis. During starvation glucagon levels are high and CAT_I activity is stimulated, on refeeding, glucagon levels fall and insulin levels rise causing inhibition of CAT_I (McGarry and Foster, 1980).

1.2 Catecholamines and the heart

Adrenaline and noradrenaline are naturally occurring catecholamines which mediate a variety of cellular functions. These hormones are released in response to stress and cause changes in both the physiology and biochemistry of the animal.

In heart, noradrenaline (released on stimulation of sympathetic nerves) and circulating adrenaline (produced by the adrenal gland) cause an increase in cardiac output. This involves an increase in both heart rate (chronotropy) and force of contraction (inotropy). To meet the increased energy demands, catecholamines also increase energy production

by myocardial metabolism. These hormones act on α- and β-adrenergic receptors at the cell membrane to generate intracellular 2nd messengers which mediate the cellular response of the hormone.

a) β-adrenergic receptors

Stimulation of β-receptors generates cyclic AMP (cAMP) (review Hayes, 1986). The pathway of cAMP formation is well established and is outlined in Figure 1.9. cAMP mediates a variety of cell responses by activating cAMP-dependent protein kinase (cAMP-PrK) which in turn phosphorylates and thereby changes the activity of numerous enzymes, including those involved in both muscle contraction and energy metabolism. A more detailed description of the effects produced by cAMP-PrK, relating to the work of this thesis, is given in subsequent sections.

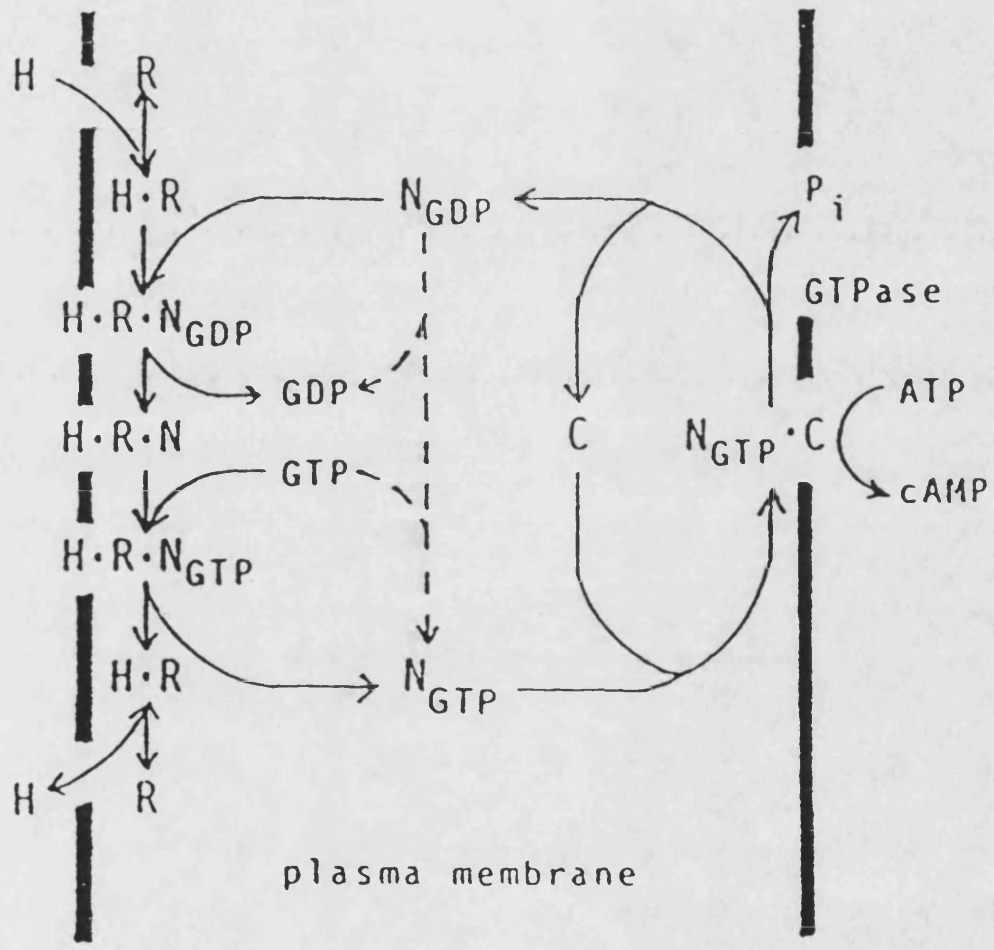
b) α-adrenergic receptors

α-adrenergic receptors have been divided into two subtypes - α_1 and α_2. Stimulation of α_2 receptors has essentially the opposite effect of that of β-receptors; deactivation of adenylate cyclase producing a reduction in cAMP levels (Jakobs et al., 1981).

More recently it has been established that activation of α_1-receptors produces two 2nd messengers - inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (review, Berridge 1987). IP_3 produces an increase in $[Ca^{2+}]_i$ and DAG activates protein kinase C (PKC).

Figure 1.9

ß-Adrenergic receptor coupling to adenylate cyclase



H= beta agonist
R= beta adrenergic receptor
N= nucleotide binding protein
C= adenyl cyclase

i) α_1-regulation of Ca^{2+} release

The pathway of α_1-induced formation of 2nd messengers is shown in figure 1.10. Binding of agonist to the receptor triggers GTP-dependent activation of phospholipase C which hydrolyses membrane phosphoinositides - hydrolysis of phosphoinositoldiphosphate (PIP_2) results in formation of IP_3 and DAG. IP_3 binds to a site on the endoplasmic reticulum to induce release of Ca^{2+} (Figure 1.11). IP_3 may then be used to re-form phosphoinositides or further metabolized to IP_4. IP_4 has been implicated in a sustained entry of Ca^{2+} from outside the cell. IP_4 can be further phosphorylated, but the role of these isomers is not yet known. When the agonist is removed, IP_3 and IP_4 levels decline rapidly thus reducing $[Ca^{2+}]_i$. (review Berridge, 1987).

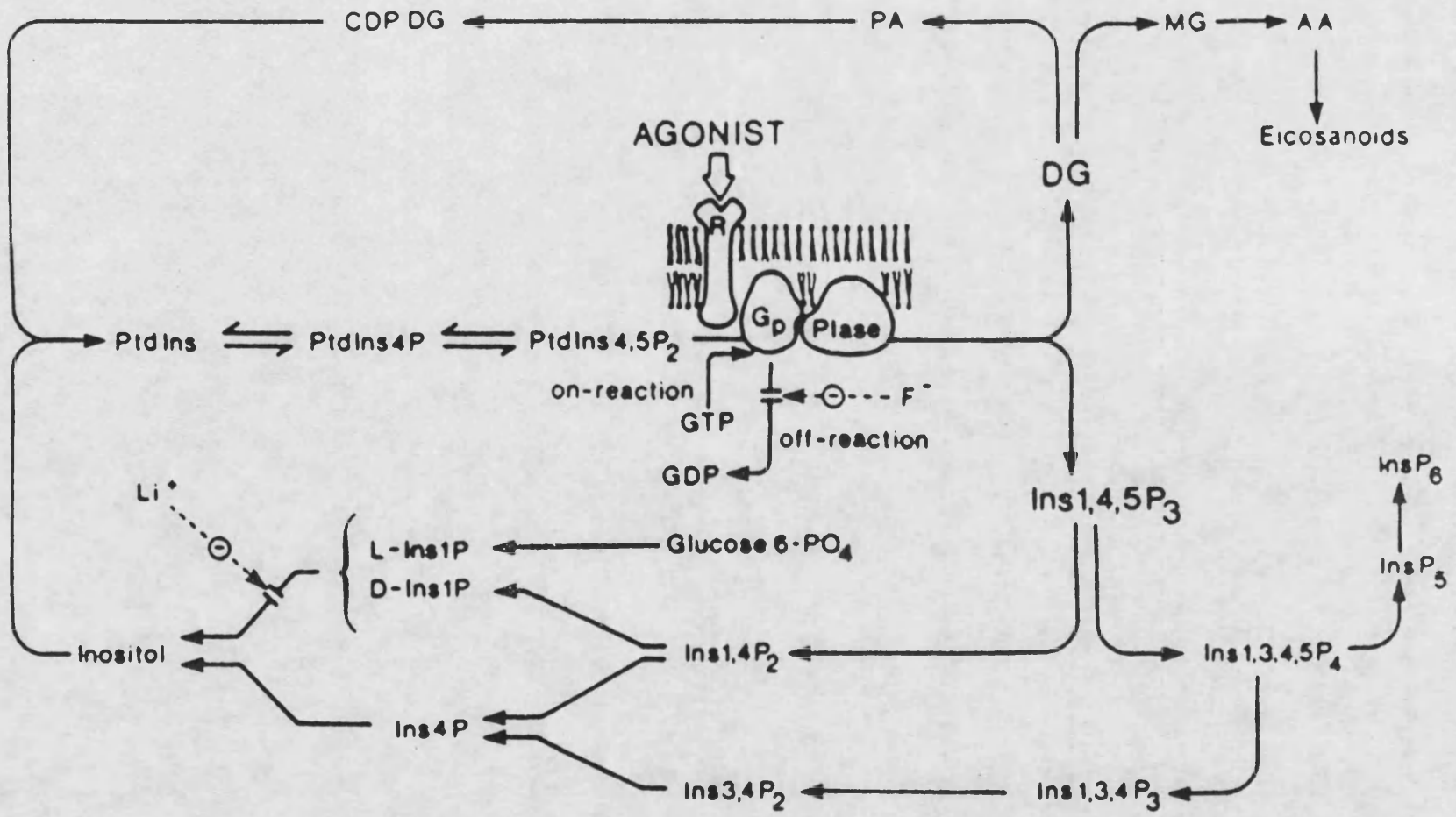
α_1-induced stimulation of phosphoinositide turnover was first shown to occur in heart muscle by Brown et al. (1985).

ii) α_1-induced activation of protein kinase C (PKC)

α_1-activation induces DAG formation which then activates PKC (Figure 1.11) following stimulation of cells, PKC is translocated from the cytosol to the membrane. Phospholipid (particularly phosphatidylserine - PS) and Ca^{2+}, as well as DAG, are required for optimal activation of PKC (review Nishizuka, 1986). PKC has been purified from heart (Kuo et al., 1984) and several *in vitro* substrate proteins

Figure 1.10

Receptor coupling to phosphatidylinositol turnover - formation of IP_3 and DG



Legend to Figure 1.10

Ptd Ins - phosphatidylinositol

Ins-1-P - inositol-1-phosphate

Gp - guanine nucleotide binding protein

Phase - phospholipase C

DG - diacylglycerol

MG - monoacylglycerol

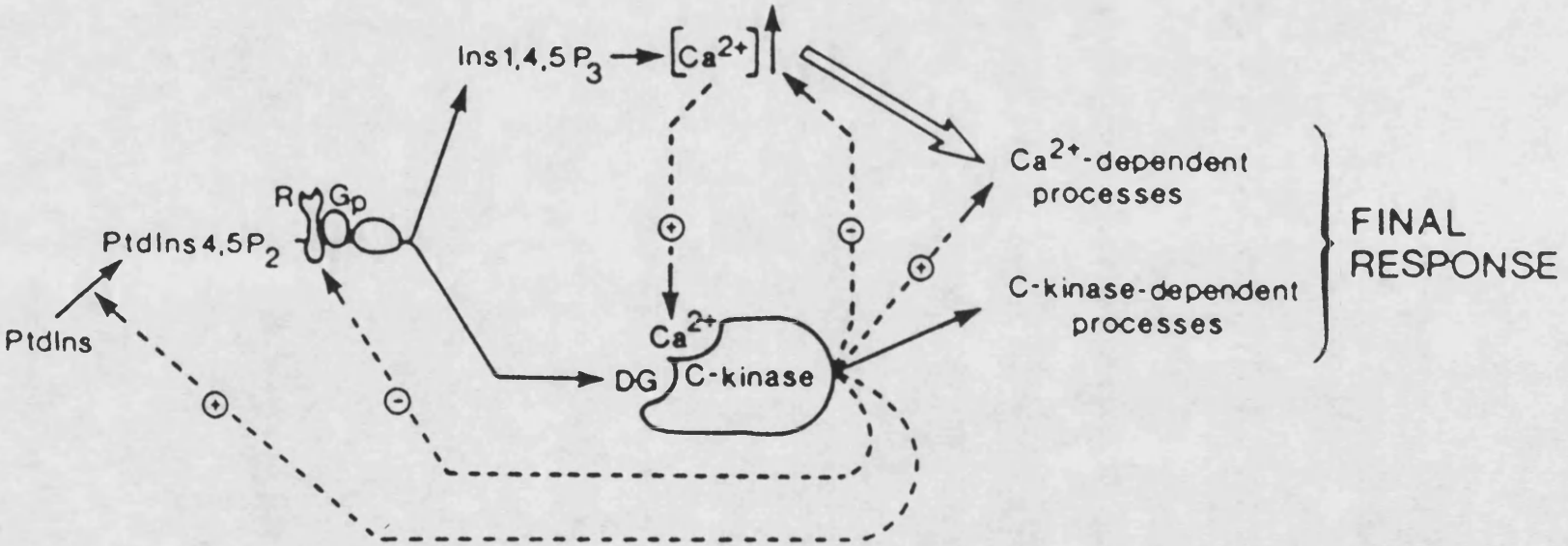
AA - Arachidonic acid

PA - phosphatidic acid

Figure 1.11

Dual signal response to phosphatidyl inositol turnover

(from Berridge, 1987)



identified (Iwasa and Hosey, 1984; Yuan and Sen, 1986) e.g. troponin-I, troponin-T and phospholamban (Movesesian et al., 1984).

iii) Phorbol esters

Phorbol esters have a structure very similar to that of DAG and can activate PKC directly *in vivo* and *in vitro* (Castagna et al., 1982). The main difference between phorbol esters and DAG is that DAG is present only transiently following α_1-stimulation, but phorbol esters are not degraded by cells. Therefore, phorbol esters may prolong the response and give a distorted picture of the normal sequence of events.

Phorbol esters have been found to decrease contractility in cultured chick heart cells (Leatherman et al., 1987) and isolated perfused rat heart (Yuan et al., 1987). However, exactly how PKC activation exerts these inhibitory effects is not known.

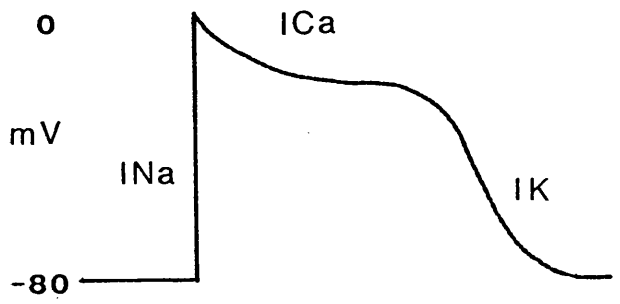
1.3 Factors controlling the level of Ca^{2+} in the cytoplasm

The calcium concentration of the myoplasm determines the activation of contractile and other proteins. The intracellular free calcium concentration ($[Ca^{2+}]_i$) in quiescent cardiac muscle is 0.1 - 0.3μM (Marban et al., 1980). Since $[Ca^{2+}]_o$ is 1 - 2mM, the Ca^{2+} level of the

cytoplasm must be controlled tightly to prevent a continuous entry of Ca^{2+} into the cell (review: Hiraoki and Vogel, 1987).

a) The cardiac action potential

Under normal conditions the heart contracts at a constant rate. Electrical impulses originate in the sinoatrial node and are conducted throughout the muscle cells in a coordinated manner to allow synchronised contraction (review, Kass and Scheuer, 1982). On reaching the cell, the impulse causes depolarization by opening voltage-dependent Na^+ channels in the sarcolemma. Na^+ ions flood into the cell to produce the inward Na^+ current (INa) which is quickly inactivated by opening of K^+ channels which allow outward K^+ movement (IK). The cardiac action potential differs from that of skeletal (and smooth) muscle in that it also contains a slow inward Ca^{2+} current (ICa) which causes a prolonged action potential (Bowman et al., 1985):



The activation of contractile proteins is initiated by Ca^{2+} release from the sarcoplasmic reticulum, however, exactly how the action potential at the sarcolemma induces such Ca^{2+} release is poorly understood. A 'Ca^{2+}-induced Ca^{2+} release' has been proposed (Fabioto and Fabioto, 1977) whereby entry of a small amount of Ca^{2+} during the action potential triggers opening of a Ca^{2+} channel in the sarcoplasmic reticulum, but other mechanisms may also operate (review, Hiraoki and Vogel, 1987).

It is not necessary to give a description of muscle contraction in this thesis, but a review can be found in Katz (1984) and England (1983).

b) Ca^{2+} entry (Figure 1.12)

Increases in $[Ca^{2+}]_i$ due to stimulation of α_1-receptors have been described in Section 1. β-receptor stimulation can also induce Ca^{2+} entry via Ca^{2+} 'slow' channels (Reuter, 1974). Catecholamines increase the probability of the channel being in the open configuration; this is probably due to phosphorylation by cAMP-PrK of a membrane protein-calciductin -which resembles phospholamban (Rinaldi et al., 1981) since phosphorylation of calciductin induced by catecholamines parallels the stimulation of Ca^{2+} slow channel activity (review, Carafoli, 1984).

Ca^{2+} uptake from the sarcoplasmic reticulum is regulated by phospholamban (review Katz, 1984), a membrane

Figure 1.12

Mechanisms regulating internal [Ca^{2+}]

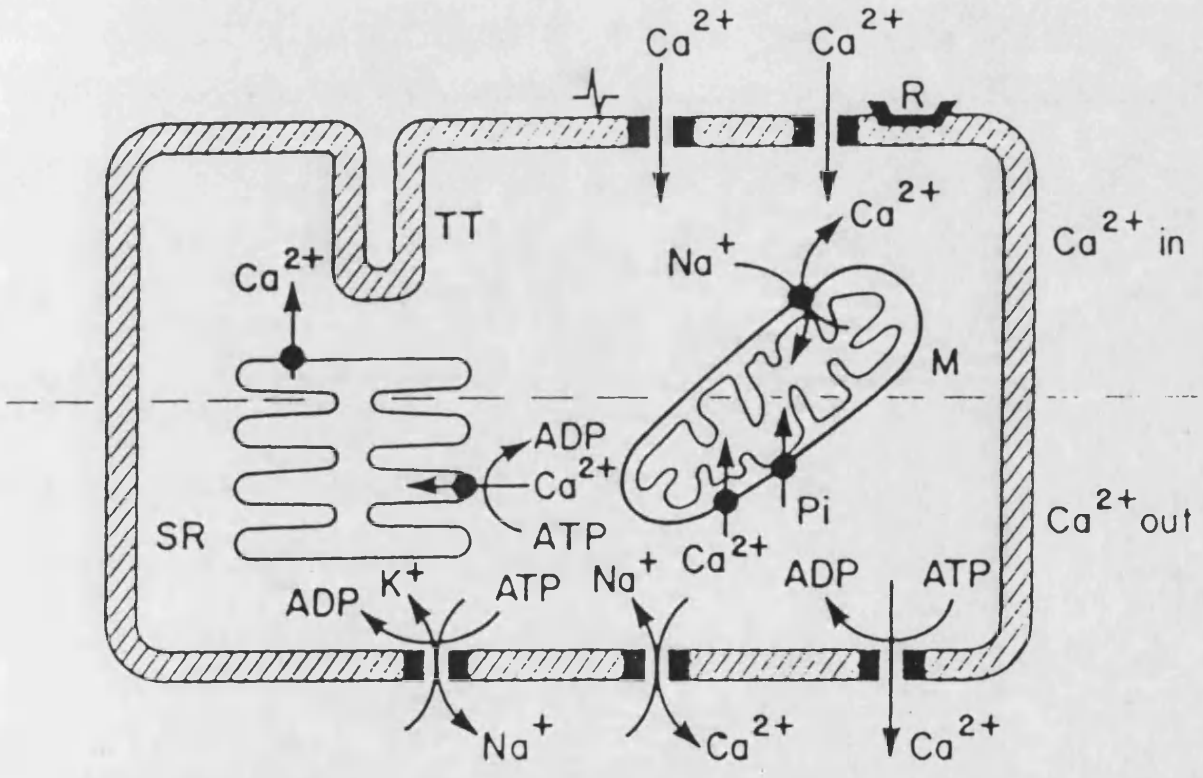
TT - transverse tubule

SR - sarcoplasmic reticulum

M - mitochondria

R and - receptor and voltage operated Ca^{2+} channels

(From Hiraoki and Vogel, 1987).



protein which can be phosphorylated by both cAMP-PrK and calmodulin-dependent protein kinase.

c) Ca^{2+} removal (Figure 1.12)

As $[Ca^{2+}]_i$ approaches 1μM, Ca^{2+}-ATPases of the sarcoplasmic reticulum and plasma membrane are activated. Ca^{2+} is thus actively removed from the cell allowing relaxation (Rasmussen, 1983). There is also an Na^+-Ca^{2+} exchanger in the plasma membrane which normally acts to remove 1 Ca^{2+} ion by allowing entry of 3 Na^+ ions (review, Inesi, 1985). Since the Na^+ gradient across the cell is established by the Na^+-K^+ ATPase (which actively pumps 3 Na^+ out of the cell whilst allowing entry of 2 K^+), the Na^+-Ca^{2+} exchange will also be influenced by ATP. If $[Ca^{2+}]_i$ rises above 1μM, the excess Ca^{2+} is absorbed by mitochondria together with Pi resulting in calcium phosphate precipitation. When $[Ca^{2+}]_i$ falls, mitochondria release Ca^{2+} through a Na^+-Ca^{2+} exchanger.

Although the functions of these membrane transport proteins are understood, details of their structure are only now emerging.

1.4 Adrenergic control of metabolism

Stimulation of the heart with catecholamines stimulates both glycogen and lipid metabolism. How catecholamines alter the activity of key enzymes in these pathways is described in the following sections.

a) Glycogen metabolism

Catecholamines stimulate glycogenolysis whilst inhibiting glycogen synthesis (review, Hayes 1986). This is summarized in Figure 1.13. Glycogen phosphorylase catalyses the initial, rate limiting step of glycogen degradation.

$$(\alpha\ 1,4\text{-glucoside})_n + Pi \longrightarrow (\alpha\ 1,4\text{-glucoside})_{n-1} + \text{glucose 1- P}$$

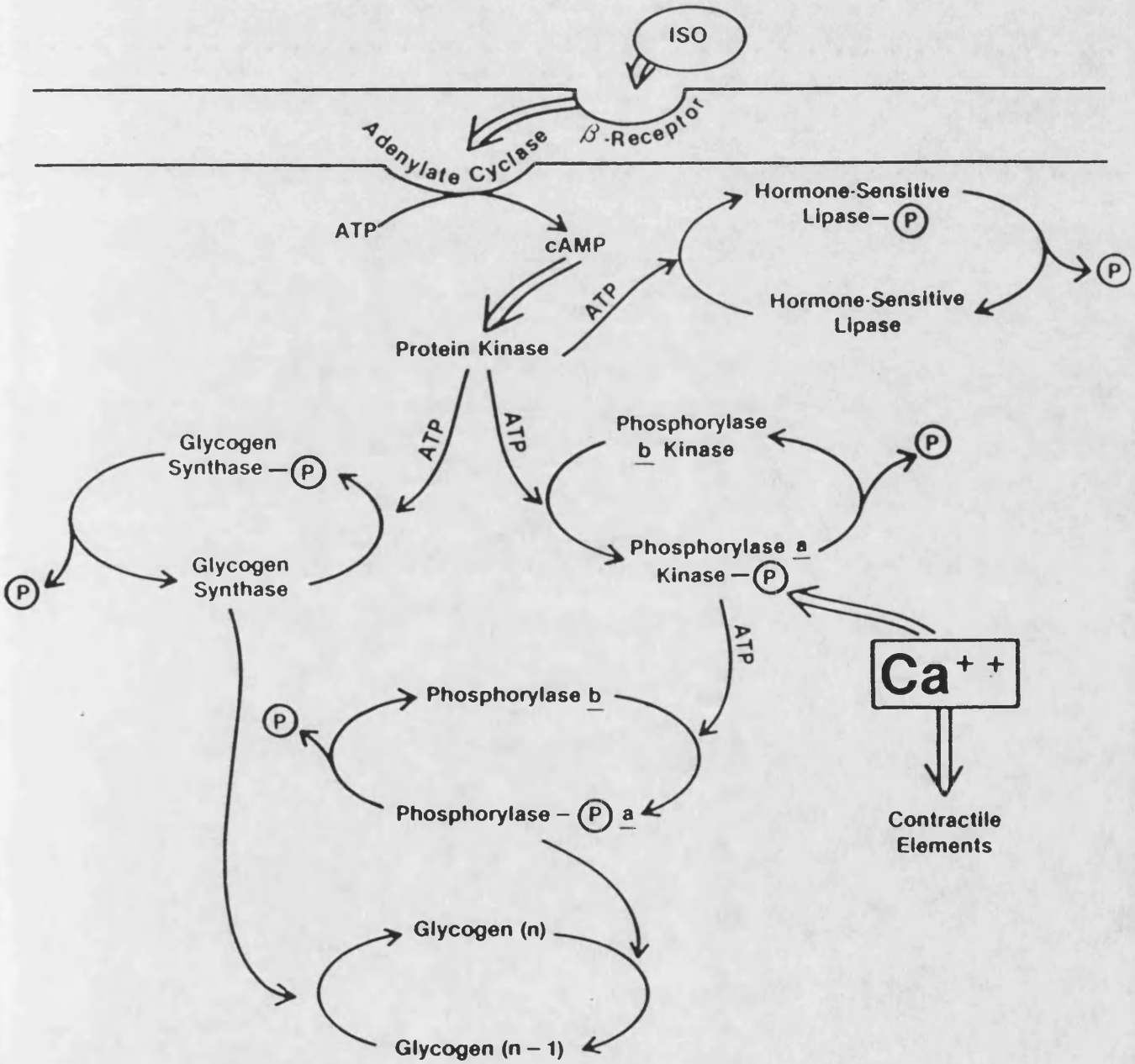
Phosphorylase exists in two forms - a phosphorylated ('a') form and dephosphorylated ('b') form. Phosphorylase 'b' is only active upon binding of 5^1-AMP, an allosteric activator (Fletterick and Madsen, 1980). Phosphorylase 'a' is also activated by AMP, but at saturating substrate concentrations is independent of the nucleotide. Phosphorylase can be inhibited by glucose, glucose-6- P and ATP.

Conversion of phosphorylase 'b' to the active 'a' form is catalysed by phosphorylase kinase (PhK). PhK contains Ca^{2+}-binding subunits which are identical to calmodulin and is thus activated by binding Ca^{2+}. PhK is also regulated by phosphorylation. Phosphorylation by cAMP-PrK activates the enzyme and also increases its sensitivity to Ca^{2+}. Dephosphorylated PhK has no activity in the absence of Ca^{2+}. (review, Hayes, 1986). Thus β-agonists stimulate phosphorylase by activating PhK by both cAMP-dependent phosphorylation and an increase in $[Ca^{2+}]_i$.

Figure 1.13

Regulation of glycogen metabolism

(from Hayes, 1986)



α_1-agonists also stimulate Ca^{2+} entry into the cell and thus activate PhK. Whether the second branch of the α_1-signalling pathway, i.e. PKC activation, is involved in activating phosphorylase will be discussed in the 'Discussion'.

Dephosphorylation and thus inactivation of phosphorylase is catalysed by protein phosphatases (review, Cohen, 1988). The activity of phosphorylase phosphatase is regulated by a protein termed inhibitor-1. Inhibitor-1 is phosphorylated and activated by cAMP-PrK to inhibit the phosphatase and thereby activate phosphorylase.

At the same time as phosphorylase is activated, the activity of glycogen synthase (GS) is inhibited by phosphorylation. GS contains at least 7 phosphorylation sites (Cohen, 1983) and can be phosphorylated by cAMP-PrK, Ca^{2+} calmodulin-dependent PrKs (including PhK) and possibly PKC (GS is a substrate for PKC in vitro - Nishizuka, 1986).

b) Regulation of lipolysis

Release of fatty acids from endogenous triglyceride is under acute hormonal control in heart (Severson, 1979): perfusing the isolated rat heart with catecholamines causes a decrease in triglyceride levels (Crass et al., 1975). The effect seems confined to β-receptors since stimulation of α_1-receptors has no measurable effect on lipolysis (Fain and Garcia-Sainz, 1983).

The rate-limiting step in triglyceride hydrolysis is catalysed by triglyceride lipase (TGL). In adipose tissue, TGL (also called 'hormone-sensitive lipase') is phosphorylated and activated by cAMP-PrK upon β-stimulation (Belfrage et al., 1980). However, the regulation of myocardial TGL is more controversial. Goldberg and Khoo (1985) and Heathers et al. (1985) found a cAMP-dependent stimulation of TGL activity in vitro. Also, Palmer et al. (1987) found that stimulation of the lipase was blocked by using a protein kinase inhibitor. However, Schoonderwoerd et al. (1987) suggested that cAMP-induced stimulation of TGL was by an indirect mechanism rather than a direct phosphorylation (this is discussed in more detail in 'Discussion'). However, most recent evidence appears in favour of a cAMP-dependent phosphorylation of TGL (Holm et al., 1988; Small et al., 1989).

Again, stimulation of TGL is confined to β-receptors since perfusion of hearts with α_1-agonists has no effect on TGL activity (Heathers et al., 1985).

As well as being under hormonal control, TGL is inhibited by fatty acids and fatty acyl CoA compounds (Severson and Hurley, 1982).

c) Control of esterification

GPAT controls the first, rate limiting step in esterification of fatty acids to triglycerides (Bell and Coleman, 1983). GPAT has been studied mainly in adipose tissue, the only published work on control of the myocardial

enzyme coming from this laboratory.

Rider and Saggerson (1983) observed a reduction in GPAT activity on incubation of rat adipocytes with noradrenaline; in whole homogenate, both mitochondrial and microsomal activities were depressed, but after isolation of the fractions, the effect persisted in the microsomal activity also. However, they found that including albumin in the homogenisation buffer removed the effect of noradrenaline, and incubation with cAMP-PrK had no effect on GPAT activity. In contrast to these results, Nimmo and Houston (1978) described an inhibition of adipose tissue GPAT with cAMP-PrK. This was initially thought to be due to phosphorylation of GPAT (Nimmo, 1981) since labelling of a band (upon SDS-PAGE of adipocyte microsomes) of Mr 54000, thought to be GPAT, occurred on incubation with cAMP-PrK and (γ^{32}P)ATP. However, later work (Nimmo and Nimmo, 1984) concluded that the labelled band differed by 500-1000 in Mr from that thought to be GPAT. The mechanism of catecholamine-induced inhibition of GPAT in adipose tissue therefore remains unsolved; it is unlikely to be due to an increase in the concentration of free fatty acids since these do not inhibit GPAT <u>in vitro</u> (Rider and Saggerson, 1983).

Perfusion of hearts with either α_1 or β-agonists causes a decrease in GPAT activity in whole homogenates (Heathers et al., 1985); this inhibition can be reversed upon incubation of heart extracts under conditions favouring activation of protein phosphatases. Also, incubation of homogenates with cAMP-PrK inhibits GPAT activity. Thus

evidence so far from heart favours a phosphorylation of GPAT by cAMP-PrK in response to β-stimulation of the heart. The mechanism of the α_1-induced inactivation of GPAT is not known and has been the subject of work carried out in this thesis.

1.5 Ischaemia

Ischaemia is defined as decreased blood flow through a tissue. Two types of ischaemia exist: chronic and acute. In man the most common cause of chronic ischaemia is atherosclerosis, a process which involves narrowing of a coronary artery by deposition of lipid and fibrous material along the lining of the artery. Flow changes resulting from such plaque formation increase the risk of both endothelial damage and platelet aggregation and thrombus formation (Azuma and Fukushima, 1976.)

Acute ischaemia is caused either by coronary thrombosis (blockage of a vessel by a fragment of solid material, usually sloughed off from atheromatous accumulations) or sudden occlusion of a coronary vessel due to a spasm of the artery. In 1912 a connection was established between coronary artery obstruction, anginal pain and sudden death (Herrick, 1912). The majority of thrombi occur in the left anterior descending, right and circumflex coronary arteries (Silver et al., 1980).

Chronic ischaemia is difficult to study experimentally due to the time required to establish the condition and nature of the causative factors. However, several methods can be used to study acute ischaemia; occlusion *in situ* of coronary vessels in dog hearts (Braunwald et al., 1974), occlusion of coronary vessels in the isolated perfused rat heart (Kannengeiser et al., 1975) or reduction in flow to the whole heart (global ischaemia).

a) Arrhythmias - general

The majority of people who die during a heart attack do so soon after the onset of symptoms (Riemersma, 1987). Abnormalities of the heartbeat which occur during ischaemia or subsequent reperfusion account for at least half the deaths due to acute myocardial infarction (Armstrong et al., 1972). Early arrhythmias occur within minutes of occlusion (Kaplinsky et al., 1979) and are the major cause of sudden death; late arrhythmias may appear days or weeks after the infarction and can usually be controlled by drugs.

The mechanisms involved in the genesis of early arrhythmias differ from those of late arrhythmias. Early arrhythmias may be due to metabolic and ionic disturbances occurring immediately after the onset of ischaemia (Corr and Sobel, 1979); this is discussed below.

b) Carbohydrate utilization and energy status

Ischaemia results in a reduced delivery of substrates and oxygen to the cell which causes a shift from aerobic fatty acid oxidation to anaerobic glycolysis (glycogenolysis is stimulated to provide glucose). The falling ATP levels produce an increase in the concentrations of ADP, AMP and P_i; this activates phosphofructokinase and hence increases glycolytic flux (Newsholme, 1972). Oxygen deficiency also increases the NADH/NAD^+ ratio causing inhibition of pyruvate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase (Neely and Morgan, 1974).

However, as lactate (the major end product of anaerobic glycolysis) accumulates, both glycolysis and glycogenolysis are inhibited (Rovetto et al., 1975); lactate also causes a drop in intracellular pH which contributes to the inhibition of glycolysis (Neely and Morgan, 1974).

Thus very little glycolytic ATP can be produced during ischaemia and the level of phosphocreatine falls in an attempt to maintain ATP levels (Garlick et al., 1979). After approximately 15 mins. ischaemia, ATP levels have fallen by over 70% (Jennings et al., 1985) and the adenine nucleotide pool (ADP, AMP) is rapidly degraded to inosine and then to hypoxanthine and xanthine.

c) Ionic changes

The membrane channels controlling the fluxes of Na^+, K^+ and Ca^{2+} in non-ischaemic myocardial cells have been described (Section 1.3). Efflux of K^+ via the Na^+ K^+ ATPase is normally inhibited by ATP but during ischaemia when ATP levels fall, the channel is activated (Noma, 1983). This, in turn, disrupts Na^+-Ca^{2+} exchange which, together with the reduced activity of the Ca^{2+}-ATPase (which normally actively pumps Ca^{2+} out of the cell) results in an increase in $[Na^+]_i$ and an increase in $[Ca^{2+}]_i$. The consequences of the increase in Ca^{2+} level are described in Section 1.6.

d) Catecholamines and the ischaemic heart

i) Catecholamine release

Increased adrenergic activity occurs in the acutely ischaemic myocardium (Carlsson et al., 1985) which may be due to activation of sympathetic nerves (Karlsberg et al., 1979) or local cardiac reflexes (Bosnjak et al., 1979). Also, direct effects on adrenergic neurones, independent of nerve impulses, could induce noradrenaline release or inhibit reuptake mechanisms. For example, an increase in extracellular K^+ concentration can induce noradrenaline release (Lorenz and Vanhoutte, 1975). Also an increase in blood catecholamine levels can accompany ischaemia (Ceremuzynski, 1981).

ii) Arrhythmias

The increased adrenergic activity in the ischaemic tissue stimulates β-receptors and intracellular cAMP levels rise markedly during ischaemia (Krause and Wollenberger, 1967; Opie et al., 1979). However, no clear cut relationship between cAMP levels and the development of arrhythmias can be demonstrated (Kane et al., 1985).

However, there is a large body of evidence implicating noradrenaline in the development of ischaemia-induced arrhythmias: (i) Chemical denervation by 6-hydroxydopamine abolishes ventricular fibrillation during both occlusion and reperfusion in cat heart (Sheridan et al., 1980) (ii) β - adrenergic antagonists decrease the incidence of ischaemia-induced arrhythmias (Khan et al., 1972) e.g. pre-perfusion with β-antagonists reduced the incidence of ischaemia-induced arrhythmias in isolated rat hearts (Kane et al., 1979; Campell et al., 1981) (iii) Sheridan et al. (1980) found that α_1-blockade with phentolamine reduced the incidence of both ischaemia and reperfusion-induced arrhythmias in cat, suggesting that events triggered by α_1-stimulation also contribute to arrhythmogenesis.

e) Lipid metabolism

During ischaemia, fatty acids and their metabolites accumulate due to an inhibition of fatty acid oxidation and lack of removal of metabolites normally washed away in the blood. Accumulation of such metabolites may contribute to cell damage and myocardial dysfunction occurring during

ischaemia.

i) Triglycerides

Glycerol is released in considerable amounts from ischaemic tissue (Vik-Mo et al., 1979) suggesting increased triglyceride hydrolysis. This is supported by the finding of an elevated TGL activity in ischaemic rat heart (Heathers and Brunt , 1985). However, a decreased content of triglyceride in ischaemic hearts could not be demonstrated (van Bilsen et al., 1989). It has been suggested that increased cycling of triglycerides occurs during ischaemia (Stam et al., 1987); the build up of glycolytic intermediates (such as glycerol 3-phosphate) stimulating re-esterification of fatty acids. However, this requires energy (ATP) to activate fatty acids to fatty acyl CoA, and it seems unlikely that the myocardium could spare ATP in this way. Also, Heathers and Brunt (1985) found a decreased GPAT activity in ischaemic myocardium which would inhibit re-esterification. It is possible that small changes in triglyceride levels (which would be enough to produce a measurable increase in fatty acid levels) are missed by current assay procedures, as suggested by van Bilsen et al., (1989).

The stimulation of TGL and inhibition of GPAT during ischaemia appears to be mediated by β-adrenergic receptors: Heathers and Brunt (1985) found that preperfusion of the isolated rat heart with β-, but not α_1-adrenergic antagonists prevented the ischaemia induced changes in enzyme activity.

ii) Phospholipids

Phospholipids are another possible source of the increased fatty acids in ischaemic tissue.

However, there are conflicting reports on phospholipase activity in ischaemic hyocardium: Internal calcium increases during ischaemia and has been suggested to activate PLA_2 (Saxon et al., 1984) but Bentham et al., (1987) found a reduced PLA_2 activity in ischaemic heart and suggested that inhibition by lysophosphoglycerides and acylcarnitines occurred. However, a decrease in phospholipid synthesis may occur, and a decreased lysophosphatidylcholine acyltransferase activity has been reported in pig heart, and also a decreased acyl CoA synthetase (Das et al., 1986).

As with triglycerides, no change in the phospholipid content of ischaemic myocardium can be detected (van der Vusse et al., 1987). Since levels of fatty acids (van Bilsen et al., 1987) acyl-CoA acyl carnitine (Neely et al., 1979; Shug et al., 1978) and lysophosphatidylcholine (Corr et al., 1983) have all been shown to increase, it is obvious that lipid metabolism in ischaemic tissue is impaired, but the mechanisms responsible have yet to be determined.

iii) Toxic effects of lipid metabolites

Normally, long chain acyl carnitines (LCAC) are concentrated in internal membranes with very little in sarcolemma (Knabb et al., 1985). However, hypoxia leads

to a 5-fold increase in the total [LCAC] but a 70-fold increase in the sarcolemma (Knabb et al., 1986). Pretreatment of hypoxic myocytes with 2-[5-(4-chlorophenyl) pentyl]oxirane-2-carboxylate (POCA - a specific inhibitor of carnitine acyltransferase I) abolished the electrophysiological derangements associated with hypoxia and also the increase in α_1-receptor density that occurs in hypoxic myocytes (Heathers et al., 1986). Analogous changes occur in ischaemic hearts in vivo in response to POCA (Creer et al., 1987).

A translocation of β-receptors from an intracellular light vesicle fraction to the sarcolemma of guinea pig hearts has been demonstrated (Maisel et al., 1985), but a similar translocation does not occur for α_1-receptors. Therefore, the increase in α_1-density seen during hypoxia and ischaemia may be due to exposure of latent α_1-receptors in the sarcolemma, possibly by insertion of LCAC into the membrane (Heathers et al., 1986).

The electrophysiological alterations contributing to arrhythmogenesis in the ischaemic heart occur within minutes. Therefore, implication of specific metabolites requires accumulation within a correspondingly brief time interval. (< 15 min.) (Corr et al., 1987). Lysophospholipids (LPLs) accumulate rapidly in ischaemic myocardium (Corr et al., 1986). The overall concentration of lysophosphatidylcholine (LPC) in ischaemic heart is ≃1.2mM. Exposure of tissue to

1.2mM LPC + 0.4mM albumin leads to electrophysiological alterations (Corr et al., 1979); in absence of albumin, 10-75μM is effective (Corr et al., 1981). Man and Lederman (1985) found that lowering the perfusate [Ca^{2+}] had a beneficial effect on LPC-induced arrhythmias in isolated rat heart, but the role of Ca^{2+} in the LPC-induced arrhythmias is not known. LPC also has a positive inotropic effect which may reflect a stimulation of adenylate cyclase (Sedlis et al., 1983).

LPLs are amphipillic molecules, by inserting into the lipid bilayer they can alter the physical and electrical properties of biological membranes (Fink and Gross 1984). Thus LPL-induced changes may contribute to the changes seen in ion conductances during ischaemia.

1.6 Reperfusion

The heart cannot recover from an ischaemic episode without restored coronary flow, but reperfusion itself may cause further damage (Corr and Witkowski, 1984; Manning and Hearse, 1984).

Periods of ischaemia greater than approximately 30 min. cause irreversible cell injury (review, Hess and Manson, 1984). Earlier reperfusion salvages the myocardium but severe mechanical dysfunction may persist. This delayed recovery has been termed 'stunning' (Ellis et al., 1983): ATP levels may remain depressed (De Boer et al., 1980) and

fatty acid levels remain elevated (van Bilsen et al., 1989; Hara et al., 1989).

a) Adrenergic activity and arrhythmias

Reperfusion of an ischaemic area can induce arrhythmias which occur within the first minute of reperfusion; the incidence of arrhythmias depends on the duration of the preceeding ischaemic episode, being maximal after 15 min. ischaemia in rat hearts (Mannings, 1986). Adrenergic antagonists have been shown to be beneficial in protecting against reperfusion-induced arrhythmias (Jennings, 1985 - review).

There is evidence for an increase in α adrenergic activity on reperfusion of a previously ischaemic area: Sheridan et al., (1980) found that both α and β-blockade decreased the incidence of ischaemia-induced arrhythmias, but only α-antagonists reduced the incidence of arrhythmias during reperfusion (using anaesthetized cat model). An increased α-adrenergic activity has also been reported by Stewart et al.(1980) and Sharma et al., (1983).

On reperfusion of the isolated rat heart, TGL activity returns to pre-ischaemic values but GPAT activity is further reduced (Heathers and Brunt, 1985). Preperfusion with the α_1-antagonist doxazosin, but not the β-antagonist atenolol, prevented the reperfusion-induced fall in GPAT activity. Also perfusion of normal hearts with the α_1-

agonist methoxamine inhibited GPAT with no change in TGL activity. Therefore, it appears that α_1-receptor mechanisms are responsible for the reperfusion-induced fall in GPAT activity.

b) Free radicals

Reperfusion also causes formation of oxygen-derived free radicals (McCord, 1985). Under normal conditions, free radical scavengers (catalase and superoxide dismutase) can pick up the free radicals and prevent cell damage. However, during ischaemia there is a decrease in the number of scavengers which are therefore unable to metabolize the radicals formed on reperfusion. Free radicals damage cells mainly by lipid peroxidation; unsaturated fatty acids and membrane phospholipids react to give an alkyl radical followed by a lipid-peroxy radical. This causes disorganization of membrane structure and function (Noronha-Dutra and Steen, 1982). This injury can be potentiated by amphiphiles (Mak et al., 1986) i.e. production of lysophospholipids and LCACs during ischaemia may enhance reperfusion injury due to peroxidation of the sarcolemma.

c) Ca^{2+} in ischaemia and reperfusion

Shen and Jennings (1972) were the first to report an increase in $[Ca^{2+}]_i$ during ischaemia and reperfusion of the canine myocardium which was associated with cell damage.

A summary of the consequences of altered Ca^{2+} homeostatis is given in Figure 1.14. This link between Ca^{2+} overload and irreversible cell injury also occurs in certain cardiomyopathies, the Ca^{2+} paradox and hypoxia (review, Nayler et al., 1985). An increased Ca^{2+} entry on reperfusion occurs even after relatively short periods of ischaemia and this increase could be blocked by the α_1-antagonist prazosin (Nayler et al., 1985), suggesting that initial Ca^{2+} uptake on reperfusion is mediated by α_1-receptors.

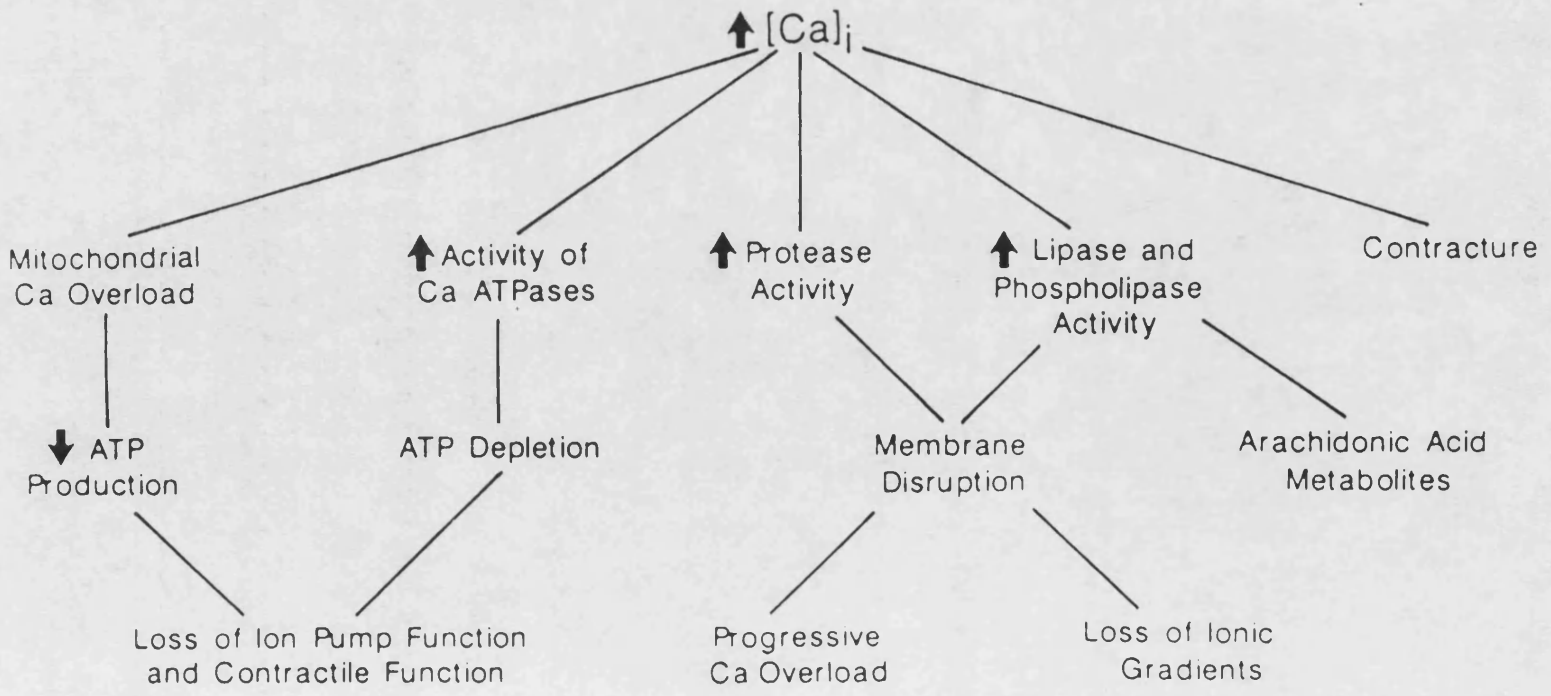
Following a period of Ca^{2+}-free perfusion (2 - 3 min.) in rat myocardium, perfusion with Ca^{2+} causes extensive cell damage. This is known as the 'Ca^{2+} paradox' (Zimmerman and Hulsmann, 1966) and the resultant cell damage is similar to that occurring on reperfusion of ischaemic myocardium (review, Hess and Manson, 1984).

The mechanism of the increased $[Ca^{2+}]_i$ during ischaemia and reperfusion is complex (review, Jennings et al., 1985) (i) local release of catecholamines may stimulate Ca^{2+} entry via the slow channels (ii) reduced ATP levels inhibit active Ca^{2+} efflux (iii) free radicals may disrupt membrane structure and increase the permeability to Ca^{2+} (Murphy et al., 1986) (iv) Ca^{2+} accumulation may be augmented by an increased α_1-adrenergic activity (review, Corr and Sharma, 1984) (v) palmitoyl carnitine accumulates during ischaemia and this can activate voltage-dependent (slow) Ca^{2+} channels

Figure 1.14

Consequences of altered Ca^{2+} homeostasis

(from Murphy et al., 1987)



(Mir and Spedding, 1986) at a concentration similar to that found in ischaemia (Spedding, 1987).

Thus attempts to preserve myocardial function during ischaemia and reperfusion by inhibiting a single pathway of Ca^{2+} influx is likely to be met with only limited success.

i) Ca^{2+} channel antagonists and arrhythmias

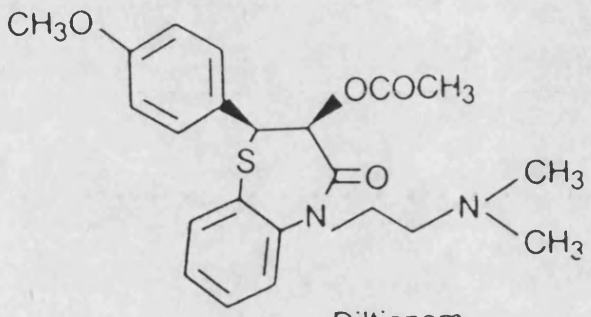
Fleckenstein and colleagues in the 1950s first established the therapeutic ability of calcium antagonism (review: Flickenstein, 1984) and the organic calcium antagonists are now used widely in the treatment of coronary artery disease: Roberts et al., (1986) demonstrated that a daily dose of diltiazem prevented the development of reinfarction in patients who had suffered an infarction.

Ca^{2+} antagonists

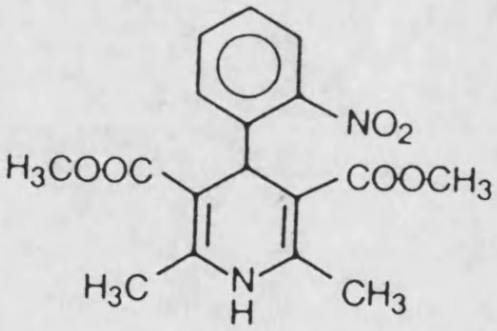
The organic Ca^{2+} channel antagonists have been divided into three subtypes based on pharmacological and structural data (review, Schwartz, 1987): (i) 1,4-dihydropyridines (nifedipine, BayK8644) (ii) phenylalkylamines (verapamil) and (iii) benzothiazipines (diltiazem). Model building from the chemical structures (Figure 1.15) revealed completely different shapes, suggesting either multiple receptor types or multiple sites on a single receptor. The slow channel has two major subunits of Mr 170 000 each (α_1 and α_2).

Figure 1.15

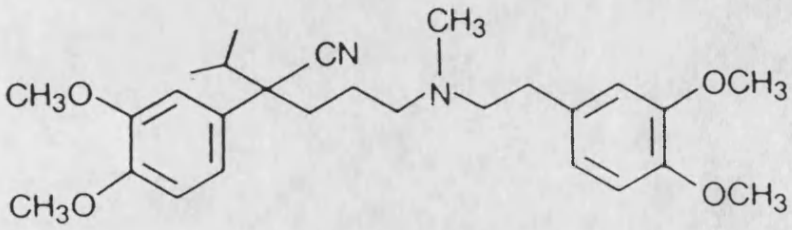
Structures of three Ca^{2+} antagonists



Diltiazem



Nifedipine



Verapamil

However, only the α_1-subunit has binding sites for the Ca^{2+} blockers (Lazdunski, 1988).

The Ca^{2+} antagonists may possess properties other than Ca^{2+} antagonism, especially at higher concentrations ($>10^{-6}$M): diltiazem and verapamil can inhibit Na^{+} channels (Bayer et al., 1975), verapamil may also have intracellular sites of action (Kaufmann, 1977) and interaction with α_1-receptors has also been reported, being most marked for verapamil and least for nifedipine (Corr and Sharma, 1984; Nayler et al., 1982). The antagonists also produce vasodilation and negative inotropy and chronotropy (Schwartz and Triggle, 1984; Braunwald, 1982).

Arrhythmias

Reperfusion-induced arrhythmias (RIAs) have been widely observed both *in vivo* (Crome et al., 1986; Sheridan et al., 1980) and *in vitro* (Lubbe et al., 1983; Manning and Hearse 1984). Ca^{2+} antagonists protect against ischaemia-induced arrhythmias (IIAs) but their ability to combat RIAs is controversial e.g. reports of verapamil being protective (Natto et al., 1981) and ineffective (Ribeiro et al., 1981) in dogs.

Interpretation is complicated when different experimental conditions are used e.g. drug dosage and administration, and also because many Ca^{2+} channel antagonists have other pharmacological properties. There is also conflicting evidence about diltiazem, whose properties are relatively

specific: ineffective against RIAs in dogs (Sheehan et al., 1982) but effective in rat (Kinoshita et al., 1987) and cat (Schwartz et al., 1986).

Tosaki et al., (1987) using isolated rat hearts, found that diltiazem reduced the rate of ventricular reperfusion induced fibrillation (VF) when given prior to occlusion, but not if administered just prior to reperfusion. This suggests that the activity of the slow channel during the early moments of reperfusion may be discounted as a determinant of the vulnerability of the heart to arrhythmias. This supports the conclusion of Marshall et al. (1981) and Manning et al. (1985) that slow Ca^{2+} antagonism *per se* does not directly influence the incidence of RIAs.

Tosaki et al.(1987) also found that the anti-arrhythmic properties of diltiazem were secondary to its ability to reduce heart rate since, if the decrease is prevented by pacing, the anti-arrhythmic properties are lost. This beneficial action of Ca^{2+} antagonists in reducing both inotropy and chronotropy has been frequently proposed (Csik et al., 1983). Bolli et al. (1986) reported a positive correlation between heart rate and the incidence of IIAs in dogs.

1.7 Diabetic myocardium

Cardiac disease is the main cause of mortality in human maturity - onset diabetes mellitis (Bradley, 1971). Although atherosclerosis of coronary arteries is a major problem (Ostrander and Epstein, 1976) there is also evidence

for a primary cardiomyopathy (congestive heart failure) in humans which cannot be explained by hypertension or valvular disease (Shapiro, 1982; Regan et al., 1977): reduced contractility (Feen et al., 1980); accumulation of periodic-acid Schiff (PAS) material in chronically diabetic dogs (+ collagen) causes increased 'stiffness' of the myocardium (Regan et al., 1974); electrophysiological abnormalities (Senges et al., 1980); ultrastructural alterations (Trach, 1976); defects in the activities of various organelles (Penparghul et al., 1981) (all observed in hearts from chemically-induced diabetic animals). A number of metabolic changes occur in diabetes and these are summarized in Figure 1.16.

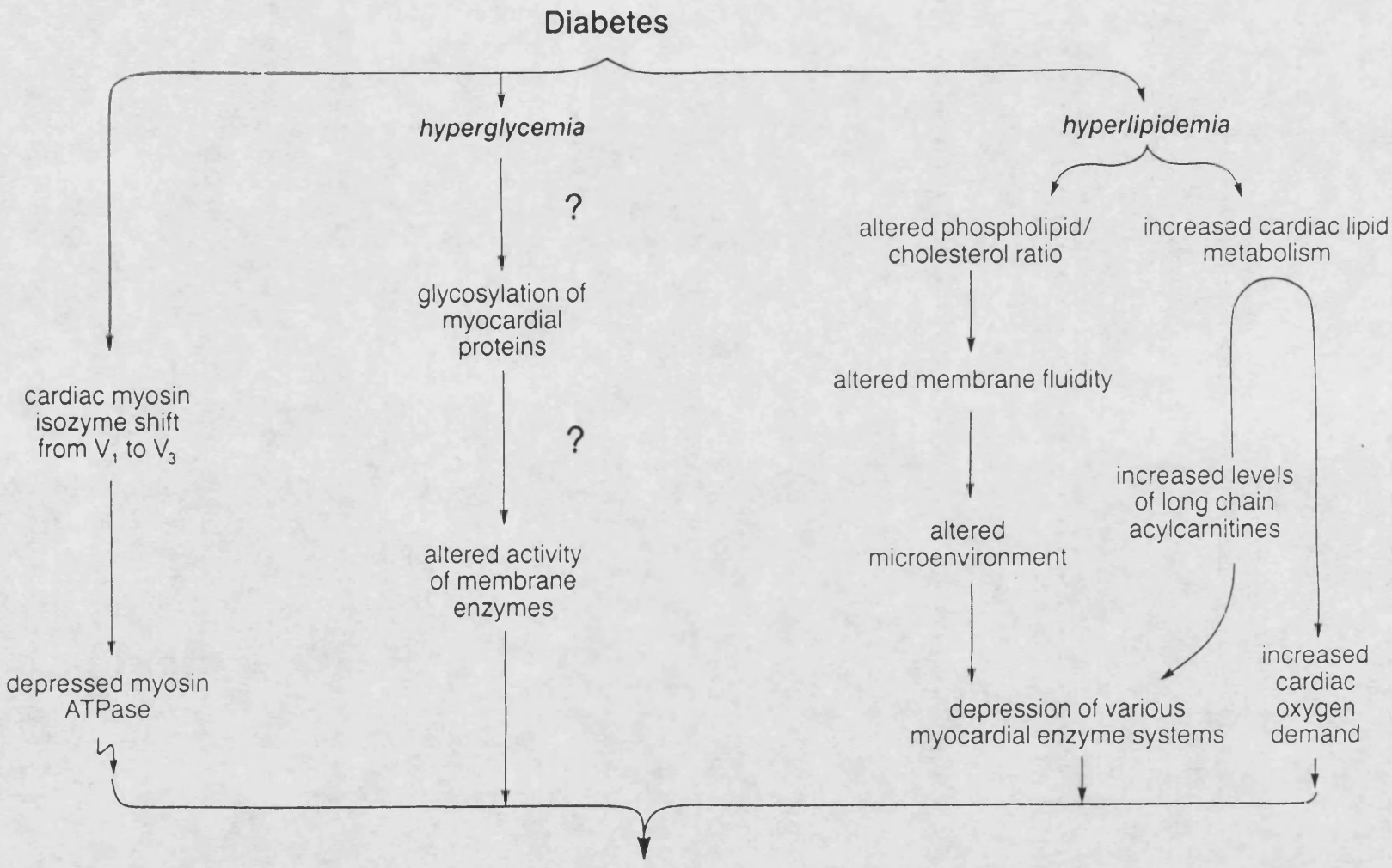
a) Animal models of diabetes

The most commonly used models for studying diabetes use either alloxan or streptozotoccin. These drugs selectively destroy β-cells of the pancreas and hence prevent insulin production. The diabetic state can be studied in either acute or chronic stages: for acute diabetes, animals are given a relatively high dose of diabetogen (> 60mg/kg for streptozotoccin) and studied 2-4 days later. In the chronic state, a lower dose of drug is used (< 60mg/kg streptozotoccin) and the animals can be studied over longer periods of time (weeks or months).

Figure 1.16

Metabolic consequences of diabetes

(from McNeill and Tahiliani, 1986).



b) Lipid metabolism

Diabetes causes an accumulation of myocardial triglycerides (Murthy and Shipp, 1977; Paulson and Crass, 1980). Levels of acyl-CoA + LCAC were also elevated and this was still evident after 60 min. perfusion with glucose as the only substrate (Paulson and Crass, 1982). Such lipid metabolites have been associated with abnormal cardiac function (rev. Corr et al., 1984).

Increased levels of circulating free fatty acids (FFA) associated with diabetes *in vivo* may lead to an accumulation of TG by increased synthesis and decreased lipolysis (Murthy et al., 1983; Paulson and Crass, 1982). They may also contribute to the increased levels of LCA-CoA and LCAC (Neely and McDonough, 1988).

Inhibition of fatty acid oxidation with POCA can normalize glucose utilization and performance in acute diabetes (Rosen and Reinauer, 1984).

c) Ischaemia and reperfusion

Increases in the levels of LCAC and acyl CoA during ischaemia have been implicated in myocardial dysfunction (Corr et al., 1984). In diabetes, there is a higher level of these metabolites, and on induction of ischaemia, a greater increase in the concentration of LCAC occurs than is seen in normals. (Feuvray et al., 1979).

In isolated perfused hearts from acutely diabetic rats, mild ischaemia was tolerated to the same extent in both normal and diabetic hearts. However, severe ischaemia caused a faster rate of ventricular failure in diabetic hearts; this was associated with a more rapid rise in the levels of acyl-CoA and LCAC (Feuvray et al., 1979).

However, reperfusion of diabetic hearts leads to a better recovery than normals: Tani and Neely (1988) exposed hearts from normal and diabetic rats to 30 min. global ischaemia and 30 min. reperfusion. Recovery (measured on developed pressure and heat rate) was better in the diabetic hearts. They suggested that this was due to a depressed Ca^{2+} uptake on reperfusion (measured using $^{45}Ca^{2+}$) of diabetic hearts.

d) Lipolysis

Perfusion of isolated hearts from diabetic rats with substrate-free or glucose only buffer produces faster rates of lipolysis compared with normal hearts. This is measured as a more rapid mobilization of radiolabelled triglyceride (Paulson and Crass, 1982; Murthy et al., 1983) or increased glycerol release (Rosen et al., 1981a). However, if the hearts are perfused under 'diabetic' conditions i.e. increased levels of FFAs, no such increase is observed (Paulson and Crass, 1982).

Regulation of lipolysis in the diabetic heart is, however, poorly understood, and conflicting results have been obtained: Rosen et al., (1981a), using acutely diabetic hearts perfused with glucose only, found a decrease lipase activity at pH7.4 (TGL) which could be restored with insulin in vivo. They also found that perfusion of isoproterenol led to the same stimulation of lipolysis (≈2 fold) in both normal and diabetic hearts. Kenno and Severson (1985), using myocytes isolated from acutely diabetic hearts, found isoproterenol produced only a small increase in lipolysis (1.2 fold compared with 2-fold in normals). Further work found an increase in neutral lipase (TGL) activity and a decrease in LPL activity (Ramirez and Severson, 1986).

e) Esterification

There are no reports on the effects of diabetes on triglyceride-synthesizing enzymes in heart. In rat adipose tissue, the activities of GPAT, MGPAT, Mg^{2+}-dependent PPH and FAS were all reduced in diabetes, whereas DGAT activity was unchanged. (Saggerson and Carpenter, 1987). Both mitochondrial and microsomal GPAT activities were depressed in this study; 2 day treatment with insulin in vivo returned FAS to control levels and GPAT, MGPAT and PPH to higher than control levels - this effect could be observed after only 2 h. of treatment. If the cells were homogenized in buffer containing albumin, the insulin-induced restoration of PPH

activity was abolished, but not that of GPAT (Taylor and Saggerson, 1986) suggesting different mechanisms are involved.

In liver of diabetic rats, no change or a slight increase in GPAT activity has been observed (Bates and Saggerson, 1977; Dang et al., 1984).

f) Adrenergic receptors

The number of β-receptors in diabetic rat heart is reduced (Savarese and Berkowitz, 1979). Heart protein yield and density of muscarinic receptors was not altered suggesting a true down-regulation of β-receptors (Ramanadham and Tenner, 1987).

There is less evidence available for α-receptors, but Heyliger et al., (1982) found a decreased response to the α-agonist methoxamine in diabetic rat papillary preparation.

During diabetes there is an increase in the concentration of circulating catecholamines (Christensen, 1974; Paulson et al., 1980). It has been shown with various cell types that long term exposure to β-agonists leads to a reduction in β-receptor number together with a decrease in responsiveness to a further challenge with the agonist (Mickey et al., 1975; Tse et al., 1979). Thus increased catecholamine levels in diabetic rats could down-regulate cardiac β-receptors and alter their responsitivity to agonists (Bitar et al., 1987).

i) Phosphorylase

Isoproterenol-stimulated adenylate cyclase activity and cAMP generation were found to be reduced in diabetic rat hearts (Atkins et al., 1985; Gotzsche, 1983). However, Vadlamudi and McNeill (1983) found that basal cAMP levels were unchanged in diabetes.

There is a general agreement that β-agonists produce a supersensitive activation of phosphorylase in hearts from acutely diabetic rats (Vadlamudi and McNeill, 1983; Miller et al., 1981; Ingebretsen et al., 1981) which may be due, at least partially, to increased Ca^{2+} levels in the diabetic heart.

g) Ca^{2+}

A number of observations have suggested an alteration in Ca^{2+} homeostasis in the diabetic myocardium. For example, diabetic hearts have a reduced inotropic response to increased $[Ca^{2+}]_o$ (Biefeld et al., 1983) and also a reduced Ca^{2+} uptake on stimulation with isoproterenol (Gotzche, 1983). In addition, the Na^+K^+ATPase and Ca^{2+}ATPase activities are depressed (Heyliger et al., 1987; Pierce et al., 1982) and the isoenzyme distribution of myosin ATPase shifts from the normally predominant V_1 to the less active V_3 form (Garber et al., 1983).

The increase in LCAC levels during acute diabetes have been found to inhibit the Ca^{2+}ATPase in isolated sarcoplasmic reticular preparations (Lopaschuk et al., 1983). Therefore, the amount of Ca^{2+} available for release may be lower than normal and contribute to the altered contraction observed in diabetic hearts. Treatment with insulin normalized LCAC levels, Ca^{2+} uptake by the sarcoplasmic reticulum and heart function (Lopaschuk et al., 1983). Carnitine treatment also normalized LCAC levels and Ca^{2+} uptake, but heart function was not improved (Vadlamudi et al., 1982). Methylpalmoxirate (an inhibitor of CAT_I) had the same effect as carnitine (Tahiliani and McNeill, 1985).

METHODS AND MATERIALS

2.1 Perfusion of hearts

a) Perfusion apparatus

The perfusion apparatus consists of a modified Langendorff system (Broadly, 1979) (figure 2.1). The system consists of water-jacketed glassware connected by polythene tubing, the perfusate being pumped by a variable speed peristaltic pump using silicone tubing at the pump.

To calibrate the flow rate, the perfusate leaving the cannula was collected in a measuring cylinder and the volume emerging recorded over a 2 minute interval. The pump speed was adjusted to obtain a flow rate of 10 mls/min. Air bubbles were removed from the system by the bubble trap.

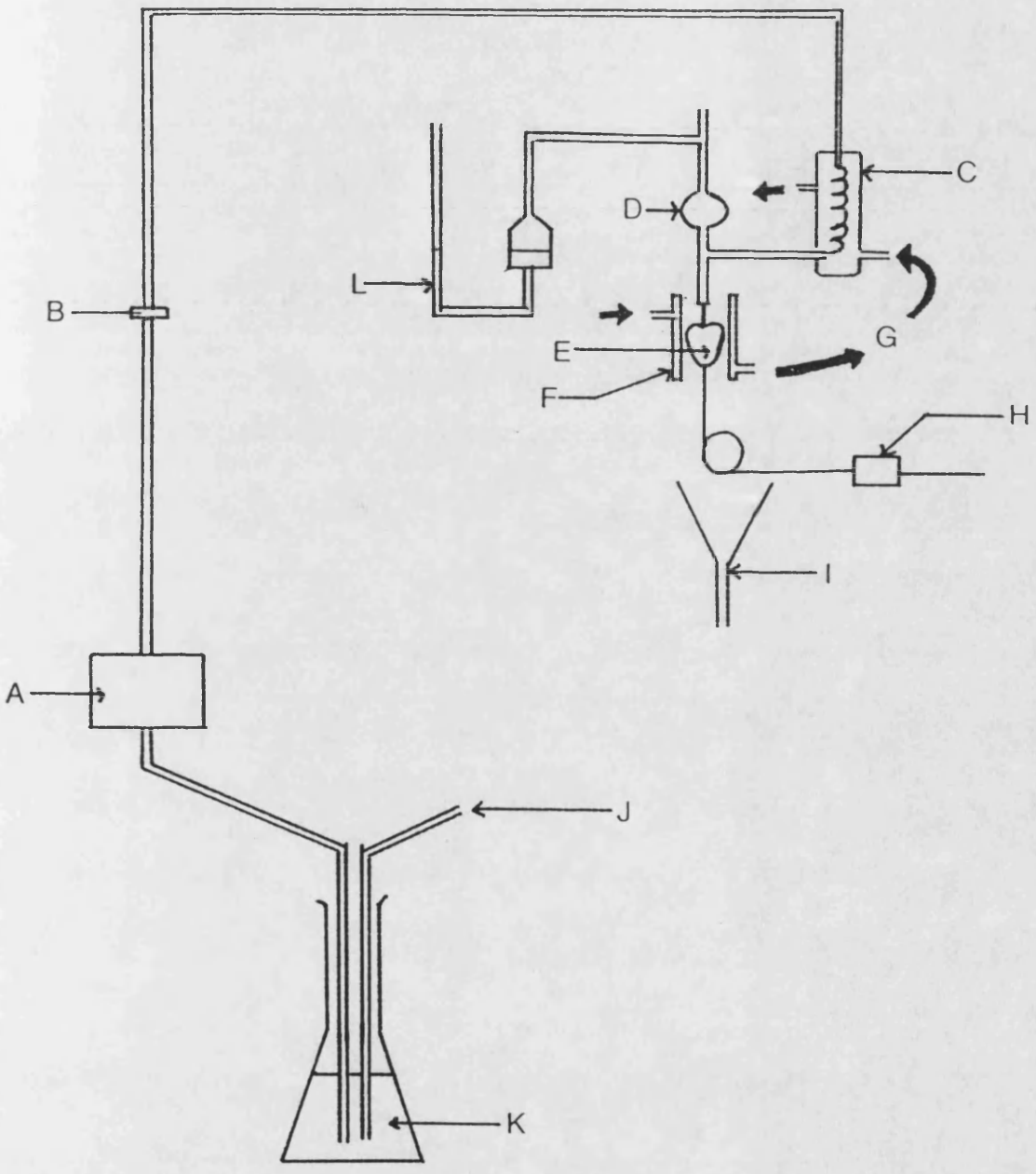
A thermostated recirculating pump supplied heated water from a reservoir to the water jacketed glassware. The thermostat was adjusted so that the temperature of the perfusate leaving the cannula was 37^{o}C. The perfusate was filtered before entering the heart by a prefilter and filter and was aerated by being gassed with 95% O_2/5% CO_2 before entering the circulation system.

b) Procedure

Male albino rats, Wistar strain, weighing 200-250g bed ad libitum on laboratory chow (Oxoid 41B) were used. Animals were stunned and their hearts rapidly removed and placed in ice-cold Krebs-Henseleit buffer (Krebs & Henseleit, 1932): NaCl - 118mM, $NaHCO_3$ - 25mM, KCl - 4.7mM, glucose - 11.6mM,

Figure 2.1

Heart perfusion system



Components of the perfusion system

A - pump

B - filter

C - heating coil

D - bubble trap

E - heart

F - water jacket

G - water at 37°C

H - transducer

I - collecting funnel

J - 95% O_2/5% CO_2

K - perfusate

L - manometer

KH_2PO_4 - 1.2mM, $MgSO_4.7H_2O$ - 1.2mM, $CaCl_2.6H_2O$ - 1.25mM, pH 7.4. This cold buffer arrested the heartbeat.

The heart was rinsed free of blood and extraneous pericardial adipose tissue removed. The aorta was attached to a vinyl cannula with an artery clip and immediately perfused with Krebs-Henseleit buffer at 37°C. The aorta was then tied to the cannula with a silk suture. Contractility was monitored with a Dynamometer UF2 transducer; a thread was attached to the apex of the heart by a small nickel hook and the thread attached to the Dynamometer by way of a pulley wheel. Developed tension of the heart could then be recorded after resting tension had been set at 2g. Heart rate was measured manually using a stop watch up to ≈250 beats/min; for higher rates the chart speed on the recorder was increased to obtain discrete beat patterns.

Following 20 min. perfusion, hearts were removed and placed in ice-cold Krebs-Henseleit buffer. The atria and aorta were transected from the ventricles and ventricular muscle was then homogenized in ≈8ml ice cold STE(F) buffer: .25M sucrose, 10mM Tris, 1mM EDTA (50mM NaF), pH 7.6 for 3 x 5s by a Polytron homogenizer (setting 3.5).

c) Perfusion procedure for studying the effects of various drugs

i) adrenaline

18.3mg adrenaline was dissolved in 100ml Krebs-Henseleit

buffer (to give a concentration of 10^{-3}M). This was then diluted to give 10^{-6}M adrenaline. 0.5ml of 10^{-6}M adrenaline was injected into the perfusion system at the point just before entry of perfusate into the bubble trap. The heart was removed 1 min. following addition of adrenaline. The solution of adrenaline was prepared just before use and discarded after 1 hour.

ii) Ca^{2+} antagonists

Diltiazem and verapamil were dissolved in Krebs-Henseleit buffer to give a concentration of 10^{-3}M. 500µl 10^{-3}M diltiazem was added to 500ml perfusate to give a final concentration of 10^{-6}M. 50µl 10^{-3}M verapamil was added to 500ml perfusate to give a final concentration of 10^{-7}M. Nifedipine was dissolved in absolute ethanol to give a concentration of 10^{-3}M. 50µl of this solution was then added to 500ml perfusate to give a final nifedipine concentration of 10^{-7}M (the final concentration of ethanol in the perfusate was .01%).

Normal perfusion was carried out for 15 min. before the perfusate was switched (by means of a 3-way tap) to one containing the required Ca^{2+} antagonist for 5 min., at which time hearts were removed.

iii) TPA

TPA was dissolved in dimethylsulphoxide at a concentration of 2 x 10^{-3}M and stored below 0^{o}C in 20µl aliquots. For perfusion studies, 380µl Krebs-Henseleit

buffer was added to the 20µl aliquot. After vortexing, the 400µl of TPA solution was added to 400ml perfusate to give a final TPA concentration of 10^{-7}M. (Gloves were worn when handling any solution containing TPA).

Hearts were perfused normally for 15 min. before switching to perfusate containing TPA for 5 min.; at this time hearts were removed.

d) Procedure for studying the effects of ischaemia and reperfusion

i) Regional ischaemia

Hearts were perfused normally for 10 min. after which time a silk suture was inserted behind the left descending coronary artery using a curved needle (length 16mm). The artery was occluded by ligation of the suture. The ischaemic area of the left ventricle is clearly visible after 10 min. occlusion at which time hearts were removed and placed in ice-cold Krebs-Henseleit buffer. Tissue samples (≈100mg) were quickly dissected from the left (ischaemic) and right (non-ischaemic) ventricles. The samples were homogenized separately in 3ml ice cold STE buffer.

For reperfusion studies, a 4mm length of polythene tubing was inserted between the artery and suture just before ligation. This tubing was removed after 10 min. occlusion allowing reperfusion of the artery. Tissue samples were taken from previously ischaemic and non-ischaemic areas at

1 min. and 5 min. after reperfusion had begun.

ii) Global ischaemia

Perfusion was carried out normally for 10 min. The pump was then switched off and a clamp (which had been previously placed just above the cannula) tightened. For ischaemic studies, the heart was removed after 10 min. For reperfusion studies, the clamp was loosened and the pump restarted. The heart was then removed after 1 min. or 5 min. of reperfusion.

e) Sterilization and washing of the perfusion apparatus

To avoid bacterial contamination the apparatus was filled with an antibiotic mixture of penicillin (300 U/ml), streptomycin (1mg/ml) and neomycin (1mg/ml) overnight. This was drained and distilled water pumped through the system before continuing perfusions.

After approximately 20 perfusions, the apparatus was disconnected and the components washed first in chromic acid and then in Decon 700 detergent. This was followed by rinsing with distilled water. At the same time all the vinyl tubing and 3-way taps were replaced.

2.2 Extraction of enzyme

After homogenization of the ventricular tissue, the homogenate was either used directly as a crude GPAT preparation or centrifuged in a bench centrifuge for 5 min. at 2000g, $4^{o}C$. The supernatant was used as a crude TGL preparation.

For preparation of mitochondria, the 2000g supernatant was centrifuged at 10 000g for 10min., 4°C (Sorvall RC 5B, 8 x 50ml head). The supernatant was kept for preparation of a microsomal fraction and the pellet washed with STE(F) buffer and centrifuged again. This pellet was resuspended in 5ml of STE(F) plus 1% defatted BSA to give a final protein concentration of 2-3mg/ml. This preparation was used for the mitochondrial form of GPAT and assay of CAT_I.

The 10 000g supernatant was centrifuged at 200 000g for 30 min., 4°C (Beckman L5-50B ultracentrifuge, SW 50.1 rotor). The pellet formed was resuspended in 0.5ml STE(F) at a protein concentration of 1.0-1.5mg/ml. This was used to assay the microsomal form of GPAT.

A portion of the 10 000g supernatant (1ml) was retained for assay of glycogen phosphorylase.

2.3 Incubation with N-ethylmaleimide

200µl of crude homogenate, mitochondrial or microsomal fraction was incubated in the absence or presence of 15mM N-ethylmaleimide (NEM) for 5min. at 30°C. Each fraction was assayed for GPAT activity. NEM-sensitive activity was calculated as that due to the microsomal form of the enzyme. NEM-insensitive activity (mitochondrial GPAT) was calculated as the difference in activity between that measured in absence and presence of NEM.

2.4 Incubation with cAMP-dependent protein kinase

Freshly perfused rat heart homogenate, mitochondrial or microsomal fraction was incubated at 30°C for 15 min. in STE buffer with, when included, 10mM $MgCl_2$, 5mM ATP, 5µM cAMP, 0.1mg cAMP-dependent protein kinase in a final volume of 200µl. Samples were assayed for GPAT activity immediately after incubation.

2.5 Incubation under dephosphorylation conditions

This method relies on the activation of endogenous phosphoprotein phosphatases by Ca^{2+} and Mg^{2+} (Severson et al 1977).

To 500µl homogenate or supernatant were added 50µl 100mM $MgCl_2$, 50µl 120mM EGTA, 50µl 120mM $CaCl_2$, 100µl 100mM MOPS, pH 7.0. The mixtures were vortexed and incubated at 30°C for 30min. Samples were assayed, for GPAT or TGL activity, immediately after incubation. Control incubations contained the same reagents except $MgCl_2$ and $CaCl_2$ (200µl water was added instead).

2.6 Incubation with TPA

Homogenate or supernatant was incubated for 15 min. at 30°C in STE buffer with, when included, 10mM $MgCl_2.6H_2O$, 5mM ATP, pH 7.6, 100µM $CaCl_2$, 10^{-7}M TPA and 20µg/ml phosphatidylserine in a final volume of 200µl. Samples were assayed immediately after incubation for GPAT and TGL activity.

These conditions favour activation of endogenous protein kinase C (Castagna et al, 1982).

i) Preparation of phosphatidylserine (PS)

5mg PS were dissolved in a small volume (≈0.5ml) chloroform which was then removed in vacuo. The precipitate was resuspended in a small volume of Tris/HCl pH 7.5 and then the volume made up to 12.5ml (this gave a PS concentration of 400µg/ml). This was then sonicated on ice for 5 min. in 15s. bursts (100w, using a 1cm probe). The solution was aliquoted out and stored at $4^{o}C$.

2.7 Diabetes

a) Induction of diabetes

Male wistar rats (200 - 250g) were starved for 24h. before induction of diabetes by a single tail-vein injection of streptozotocin (120mg/kg) dissolved in 10mM Na citrate (100mg/ml), pH 4.2. The solution was prepared immediately before use.

Hearts were isolated 48h after the injection and perfused as before.

b) Blood glucose measurement

Blood glucose levels were determined by the method of Asatoor and King (1954).

The solutions required are as follows:

Cu reagent - 3g $CuSO_4.5H_2O$ in 500ml H_2O

Hardings reagent - Dissolve in order 6g NaK tartrate, 10g Na_2CO_3, 12.5g $NaHCO_3$ 9g K oxalate in 500ml H_2O

Nelson's reagent - Dissolve 10g ammonium molybdate in 180ml H_2O. Slowly add 8.4ml conc. H_2SO_4. Dissolve in 10ml H_2O 1.2g Na arsenate ($Na_2HAsO_4.7H_2O$) then add to solution and make up to 200ml. Store at 37°C.

The procedure is as follows:

(i) Take 100µl blood and add 1.5ml H_2O, 0.2ml 0.15M $Ba(OH)_2$, 0.2ml 5% $ZnSO_4.3H_2O$. Vortex and spin at 2000 rpm. Keep supernatant. Diabetic rats will require blood to be diluted, so use only 100µl supernatant in step (iii).

(ii) Prepare glucose standards (0-100µg) using a stock solution of 0.1mg/ml, in a final volume of 1ml.

(iii) To 1ml final volume (supernatant or glucose standard) add 1ml Cu-reagent and 1ml Hardings reagent. Vortex and boil for 20 min.

(iv) Cool, add 1ml Nelson's reagent and 6ml H_2O.

Read absorbance at 600nm.

2.8 Determination of protein content

The method used was that of Bradford (1976) with bovine serum albumin as standard.

50mg Coomassie blue G-250 was dissolved in 25ml 95% ethanol. To this was added 50ml orthophosphoric acid and the volume made up to 500ml with double distilled water. The solution was filtered twice through Whatman filter paper (no. 1) before use. 2ml of this solution was added to 100µl of the test protein (<30µg) and the absorbance read at 595nm.

2.9 Determination of enzyme activities

a) Glycerol 3-phosphate acyltransferase (GPAT)

The principle of the method (Evans, 1977) is as follows:

long chain acyl CoA + G3P $\xrightarrow{GPAT}$ monoacyl G3P + CoA

monoacyl G3P + long chain acyl CoA $\xrightarrow{MGPAT}$ diacyl G3P + CoA

G3P - glycerol 3 phosphate; MGPAT - monoacylglycerol 3 phosphate acyltransferase - not rate limiting

The assay was carried out at 30°C in a final volume of 100µl containing 0.25M sucrose, 10mM Tris, 1mM EDTA 50mM KCl, pH 7.4, 0.1mM palmitoyl CoA, 0.3mg BSA (defatted) and 0.5mM [U-^{14}C] glycerol 3-phosphate (0.1µCi). The reaction was initiated by addition of 10µl homogenate (≃40µg protein), mitochondrial (≃25µg) or microsomal (≃12.5µg protein) fraction.

The reaction was stopped after 5 min. by addition of 500µl trichloroacetic acid (TCA; 10% w/v) - saturated butanol.

The solution was vortexed and the layers separated by centrifugation in an Eppendorf microfuge for 2 min. The lower aqueous layer was removed with a Gilson pipette set at 110µl and the butanol layer washed with 1ml butanol-saturated TCA. After a further centrifugation, a 100µl sample of the butanol layer was dissolved in 2ml scintillation fluid (600ml Triton, 1400ml toluene, 10g PPO). Each time point was done in triplicate. A zero time point was measured and subtracted from the measurement at 5 min. Radioactivity was counted in a Tricarb. liquid scintillation counter.

b) Triglyceride lipase (TGL)

The activity of TGL was determined by a modification of the method of Severson (1979).

The glycerol tri (1-^{14}C oleate (triolein) substrate, 9.2mM in hexane (specific activity 1.6µCi/mM) was dried under nitrogen and resuspended in an equal volume (3.125ml) of absolute ethanol. A solution of 264mg triolein in 1ml hexane was prepared and 0.1ml added to 3.025ml absolute ethanol. This was used to increase the concentration of triolein in the radioactive aliquots. 10µl 'hot' triolein and 10µl 'cold' triolein were mixed together just before use.

The assay was carried out in a final volume of 80µl containing 0.35mM ^{14}C-triolein (.16µCi) 50mM sodium phosphate and 0.08mg albumin pH 7.5. This suspension was sonicated for 30s. immediately before use. The reaction was initiated

by the addition of 20µl supernatant (≃40µg protein). Each time point was done in triplicate.

After 30 min. the reaction was stopped by the addition of 300µl of a solvent system containing methanol:chloroform: heptane (1.41:1.25:1) and 0.1mM oleic acid (which acted as carrier). 10µl NaOH was also added. The mixture was vortexed vigorously and centrifuged in an Eppendorf microfuge for 3 mins. A 50µl aliquot of the upper, aqueous layer was dissolved in 4ml scintillation fluid with 5µl HCl and then counted.

c) Carnitine palmitoyl transferase (CPT)

This method is based on that of Saggerson, (1982).

$$\text{Pal CoA + L(-) carnitine} \xrightarrow{\text{CPT}} \text{Pal-carnitine + CoA}$$

The assay was carried out at 30°C in a final volume of 100µl containing 0.25M sucrose, 10mM Tris, 50mM KCl, 1mM EDTA, pH 7.4, with 0.5mM DL-(methyl-^{14}C)-carnitine hydrochloride (.05µCi), 50µM Pal-CoA and .02mg defatted BSA. The reaction was initiated by addition of 10µl mitochondrial fraction (≃25mg protein).

After 3 min. the reaction was stopped by addition of 50µl 6M HCl; 300µl of water-saturated butanol was then added. After vortexing, the layers were separated by centrifugation in an Eppendorf microfuge for 2 mins. The aqueous (lower) layer was removed while the butanol layer was washed with

750µl butanol-saturated water. After vortexing and a further centrifugation, 100µl of the butanol (upper) layer was dissolved in 2ml scintillation fluid. Each time point was done in triplicate. Radioactivity was counted in a Tricarb scintillation counter.

d) Glycogen phosphorylase

i) Gel filtration

AMP is a potent activator of phosphorylase so in order to distinguish between activation by phosphorylation and increases in AMP concentration, endogenous AMP was removed from the extract before assaying.

A prepacked column of Sephadex G-25 (PD-10), dimensions 5 x 1cm (Pharmacia) was equilibriated in 50ml STE(F) at 4°C. The gel bed was drained and 0.85ml 10 000g supernatant layered onto the column. Once the extract had permeated the gel base, the column was eluted with STE(F). 0.9ml fractions were collected manually and the first 0.9ml eluate that contained protein (estimated by the colour difference) was used for the phosphorylase assay.

ii) Assay

Phosphorylase was assayed in the direction of glucose-1-phosphate by a spectrophotometric assay based on that of Mendicino et al (1976). The principle of the assay is as follows: Glucose 1-phosphate (Glu-1- P) produced by

phosphorylase is isomerized to Glu-6- P which is then oxidized to 6-phosphoglucano-δ-lactone by $NADP^+$.

$$(\alpha\ 1,4\text{-glucoside})_n + P_i \xrightarrow{\text{Phosphorylase}} (\alpha 1,4\ \text{glucoside})_{n-1} + \alpha\text{-D glucose 1- P}$$

$$\alpha\text{D Glu-1- P} \xrightarrow[\text{Phosphoglucomutase}]{\text{Glu 1,6 diphosphate, } Mg^{2+}} \alpha\text{D-Glu-6- P}$$

$$\alpha\text{-D-Glu-6- P} \xrightarrow{\text{Glu 6- P dehydrogenase}} 6\ \text{phosphoglucano-}\delta\text{-lactone} + NADPH + H^+$$

NADPH production is estimated at 340nm.

Enzyme assays were performed at room temperature in a Cecil CE 272 single beam uv/visible region spectrophotometer at 340nm. Absorbance changes were monitored continually on a Rikandenki chart recorder.

Assays were carried out in 1ml plastic cuvettes in a final volume of 1ml containing: 50mM imidazole, 10mM $MgCl_2$, 2mM EDTA, pH 7.0, 0.1mg/ml BSA, 0.4mg/ml protamine sulphate, 70mM potassium phosphate, 0.3mM $NADP^+$, 10μM Glu 1, 6 diphosphate, 3.26 μmol/min Glu 6 P dehydrogenase, 4.9 μmol/min phosphoglucomutase, 0.3mg/ml glycogen and 1mM AMP.

All components, except AMP, were added to the cuvette. The reaction was initiated by the addition of 10µl extract (≈15µg protein). After the AMP-independent rate had been obtained, AMP was added to the cuvette and the new rate measured. This gave an estimate of total phosphorylase (a and b) activity.

2.10 Calculation and analysis of results

Statistical analysis was performed using Student's t-test. Values are presented as means ± standard error (S.E.).

a) GPAT

GPAT activity is expressed as nmole/min/mg protein

$$\frac{\text{sample count}}{\text{standard count}} \times \text{nmoles in standard} \times \frac{1}{\text{time (min)}} \times \frac{1000}{\mu\text{g protein in aliquot}} \times \frac{500}{100}$$

Standard count was measured by taking 10 µl from the assay mixture, adding 2ml scintillation fluid and counting.

b) TGL

TGL activity is expressed as nmole/min/mg

$$\frac{\text{sample count}}{\text{standard count}} \times \text{nmoles in standard} \times \frac{1}{\text{time}} \times \frac{1000}{\mu\text{g protein in aliquot}} \times \frac{210}{50}$$

Standard count was measured by taking 10µl from the assay mixture, adding 4ml scintillation fluid, vortexing and counting.

c) CPT

CPT_1 activity is expressed as nmole/min/mg

$$\frac{\text{sample count}}{\text{standard count}} \times \text{nmoles in standard} \times \frac{1}{\text{time}} \times \mu g \frac{1000}{\text{protein}} \times \frac{306}{100}$$

To calculate the standard count, 10µl of the assay mixture was added to 2ml scintillation fluid, vortexed and counted.

d) Glycogen phosphorylase

Phosphorylase activity is expressed in terms of rate in the absence of AMP/rate in presence of AMP (the -AMP/+AMP ratio) which is proportional to the amount of phorphorylase a.

In order to obtain an estimate of the -AMP/+AMP ratio the enzyme was incubated at $0^{o}C$ and assayed at various intervals (≈8 min.) for 3-4h. following removal of the heart from the perfusion apparatus.

It was found that -AMP/+AMP ratio declined even with time. It was assumed that this linear decline had been constant since heart homogenisation and the initial rate was calculated by back extrapolation using linear regression.

2.11 Materials

a) Equipment

.45µm membrane filters were obtained from Sartorius GmbH, Gottingen, West Germany or from Millipore, London. 2.5µm

prefilters were from Millipore. The membrane filter housing was a Millipore Swinnex 25.

The peristaltic perfusion pump was from Watson Marlowe, Falmouth, U.K. Silicone tubing for the pump and glass to polythene tubing connections were from Watson Marlowe, Falmouth, Cornwall - internal diameter 3.2mm, external diameter 6.4mm. Polythene transmission tubing (i.d. 0.86mm, o.d. 4.8mm) was supplied by Portex Ltd., Hythe, Kent.

Perfusion glassware was obtained from Jencons Ltd., Hemel Hempstead, Herts. Physiological recorders were from Devices Instruments Ltd., Welwyn Garden City, Herts.

Tricarb liquid scintillation counter was from Packard Instruments Ltd., Caversham, Berks. Polytron homogenizer was obtained from Northern Media Supply Ltd., North Humberside.

Sorvall RC-5B refrigerated superspeed centrifuge was obtained from Dupont Company, Connecticut, U.S.A. Beckman L5-50B ultracentrifuge was from Beckman Instruments, California, U.S.A.

Sephadex G.25 columns were supplied by Pharmacia-LKB, Uppsala, Sweden.

b) Chemicals

Laboratory chemicals were Analar grade from BDH Ltd., Poole, Dorset.

Radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks.

All drugs, enzymes, substrates and cofactors were supplied by Sigma Chemical Company Ltd., Poole, Dorset, except for glycogen (AMP-free) and NADP (disodium salt) which were from Boehringer-Mannheim, Lewes, U.K.

2.12 Characteristics of enzyme assays

a) Triglyceride lipase (TGL)

The heart contains several lipase activities (Severson, 1979; Stam et al., 1986); an acid lipase can be distinguished easily on the basis of its pH optimum (4.8). However, it is more difficult to distinguish between endogenous lipoprotein lipase (LPL) activity and neutral triglyceride lipase (TGL) activity. Work from this laboratory (Al-Muhtaseb, 1982) and elsewhere (Severson, 1979; Ramirez et al., 1985) has identified a lipase with properties distinct from those of LPL.

The assay for TGL activity used in this thesis was developed by Al-Muhtaseb (1982). The major characteristics, which are in general agreement with those found by Severson (1979), are summarized below.

i) Assay buffers

Optimal assay conditions were as follows: TGL - phosphate buffer and 1% BSA, pH 7.5 (inhibited by serum; LPL - tricine buffer with 1% BSA, pH 8.2 (stimulated by serum).

ii) Protamine sulphate

1mg/ml protamine sulphate stimulated TGL activity and inhibited LPL activity.

iii) Heparin

Heparin had no effect on TGL activity, but at 20µg/ml stimulated LPL activity.

iv) Chloroquine

TGL activity was stimulated at both 1 and 15mM chloroquine. At higher concentrations (> 20mM) it was inhibited. LPL activity was essentially inhibited at all concentrations of chloroquine.

v) NaCl

.06M to .4M NaCl had no effect on TGL activity, but at .6M NaCl it was inhibited. LPL activity was inhibited at all concentrations of NaCl.

vi) Effect of substrate concentration

Increasing the concentration of (^{14}C) triolein increased the rate of reaction up to .46mM. This gave a Km of .4mM.

vii) Effect of incubation time and enzyme concentration

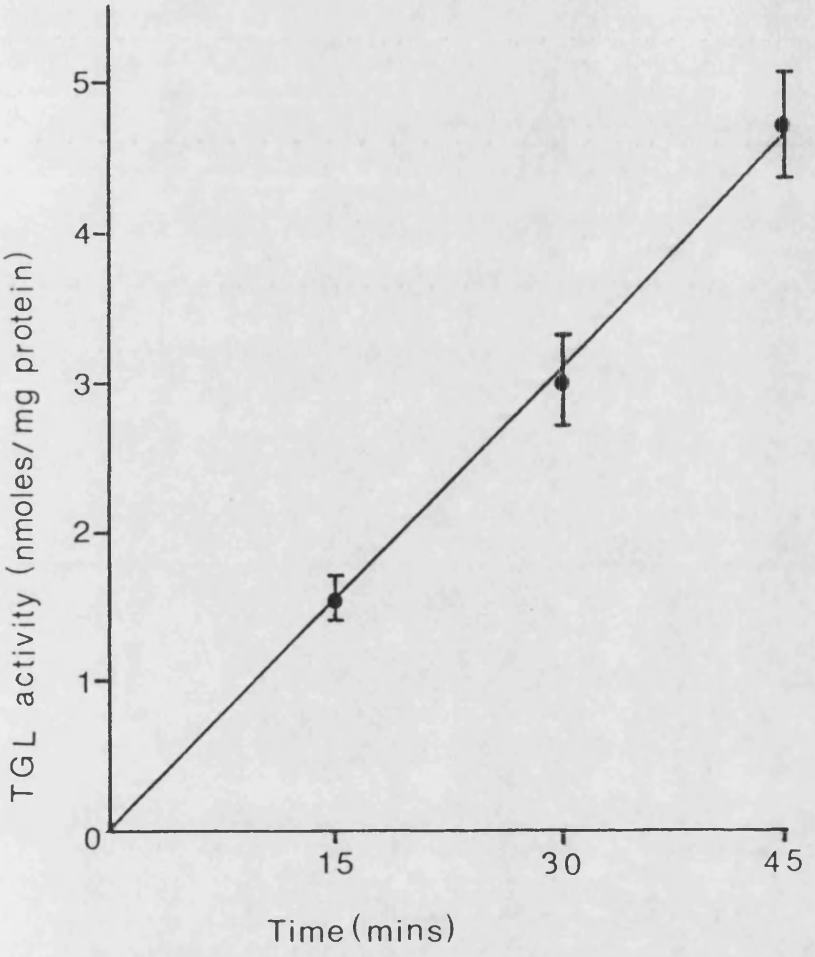
Figure 2.2 shows that the TGL assay is linear over 45 min. In subsequent assays the enzyme activity was measured at 30 min.

Figure 2.2

Effect of incubation time on TGL activity

Number of observations at each point = 6

Values are means $\pm$ S.E.



Heathers et al. (1985) showed that the assay was linear up to at least 100µg protein per assay. In subsequent assays 20-40µg protein was added per assay.

b) Glycerol 3-phosphate acyltransferase (GPAT)

In heart and other tissues, triglyceride synthesis occurs on the outer surface of the reticular system (endoplasmic or sarcoplasmic reticulum) (Bell and Coleman, 1983). Myocardial GPAT has been studied in this laboratory for several years. Other workers have focussed on adipose tissue and hepatic GPAT (Nimmo and Nimmo, 1981; Rider and Saggerson, 1983). Generally it has been assumed that the triglyceride synthesis pathway in heart is similar to that of adipose tissue.

The assay method employed here is that described by Al-Muhtaseb (1982) using (^{14}C)-glycerol-3-phosphate and palmitoyl-CoA as substrates.

i) Effect of incubation time and enzyme concentration

GPAT activity was measured in ventricular homogenates. The assay was linear with time up to only 6 mins. (Figure 2.3). Possible reasons for this are exhaustion of one of the substrates or end product inhibition. An incubation time of 5 min. was adopted as routine in subsequent assays on homogenate.

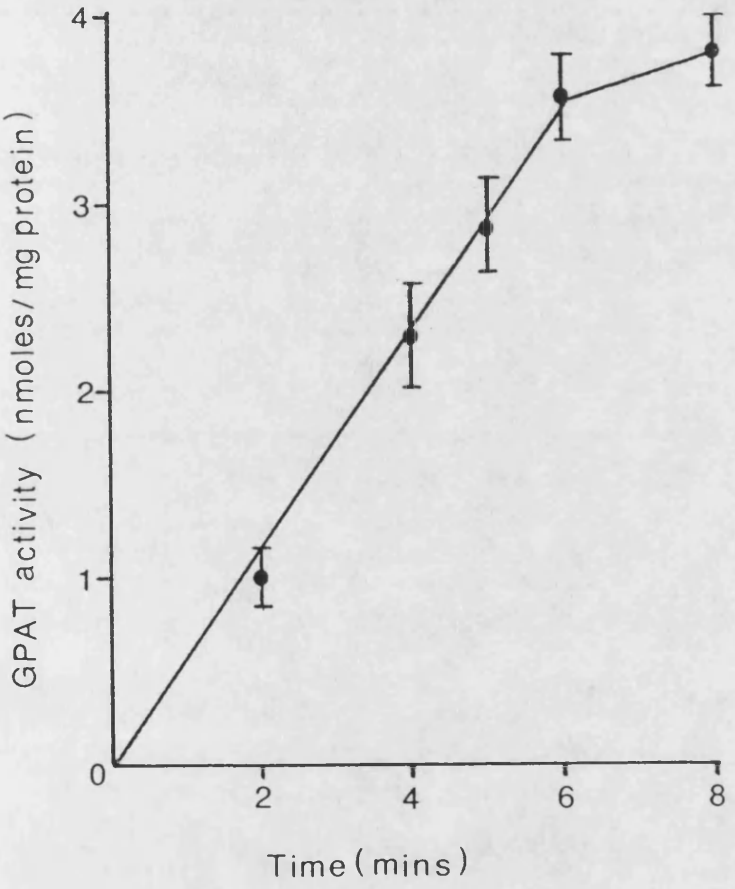
Figure 2. 3

Effect of incubation time on GPAT activity

Number of observations at each point = 6

Values are means $\pm$ S.E.

(GPAT activity was measured in crude homogenate)



Heathers et al. (1985) found that assays were linear up to at least 130µg protein per assay. Subsequently, 50-70µg protein was used per assay.

ii) Effect of substrate and albumin concentration

Concentrations of palmitoyl CoA (pal CoA) up to 100µM increase the rate of reaction (Al Muhtaseb, 1982) but above this concentration the rate decreases slowly. A Km of 50µM was obtained.

Increasing [^{14}C-G3P] up to 1mM increased the reaction rate giving a Km of 0.5mM.

[BSA] up to 2mg/ml stimulated GPAT activity but at higher concentrations the rate declined slowly (Al-Muhtaseb, 1982).

iii) Identification of lipid end products

Al-Muhtaseb (1982) found that the majority (79%) of the end product was phosphatidate with little accumulation of triglyceride or monoacylglycerol phosphate. This was studied by thin layer chromatography of whole heart homogenate.

c) Phosphorylase

The rate of reaction was found to be linear with time up to at least 10 min. This rate was dependent on the presence of all assay components except AMP (which stimulated the measured rate) and protamine sulphate (which was included to inhibit phosphorylase phosphatase - Ball and Fischer, 1986). The assay rate was proportional to protein concentration over the range used (< 30µg/assay). Subsequently, ≈20µg protein was added per assay.

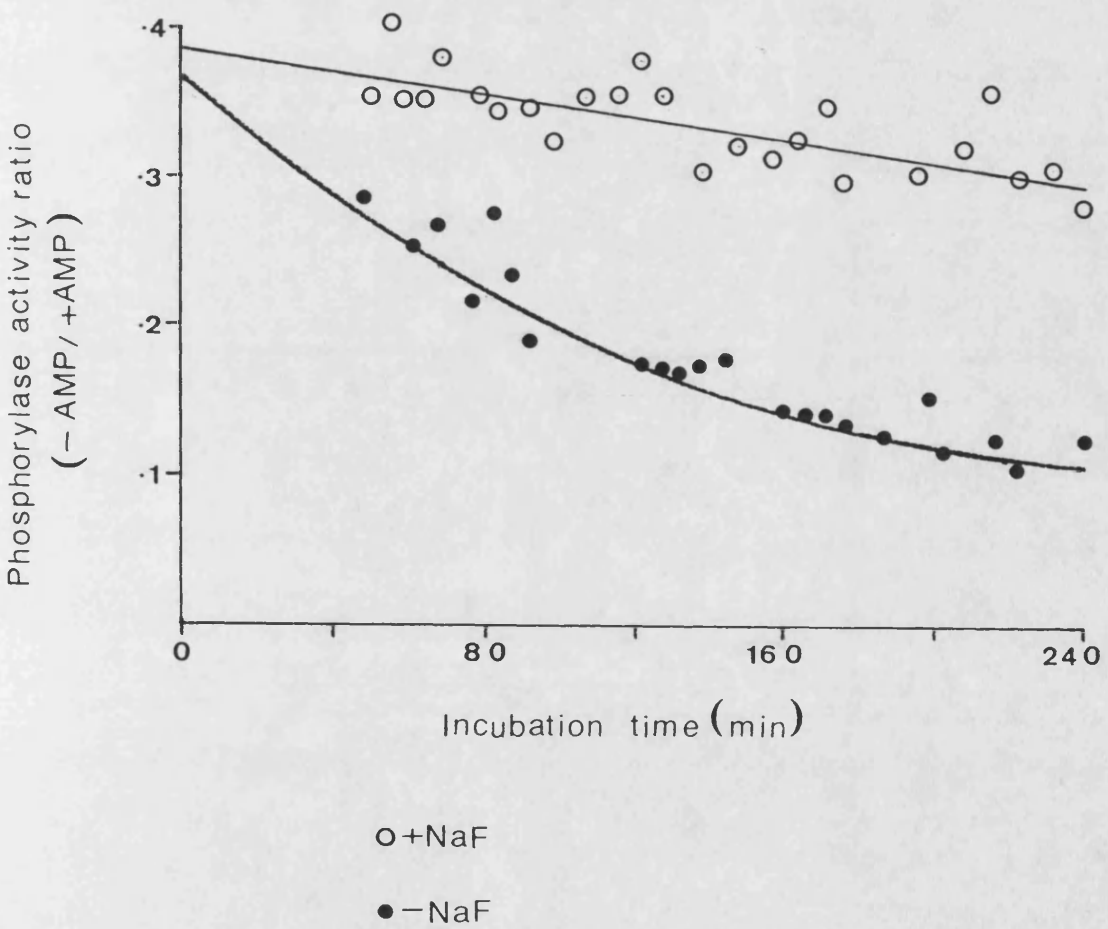
The phosphorylase activity in extracts of hearts homogenised in STE declines at $0^{o}C$ and this decline was markedly curved (Figure 2.4). Inclusion of 50mM NaF in the homogenization and gel filtration buffers reduced the magnitude of the decline and produced a linear rate (Figure 2.4). Since NaF is a phosphatase inhibitor, this result suggests that the decline in activity observed in hearts homogenised in STE was due to endogenous phosphatase action on phosphorylase. Since analysis of results by linear regression was possible, NaF was routinely included in phosphorylase extraction buffers.

(N.B. Results in Figure 2.4 are from hearts treated with 1µM adrenaline.)

Figure 2.4

Effect of NaF on phosphorylase activity

Adrenaline-treated hearts were homogenised in absence or presence of NaF and phosphorylase activity determined following gel filtration as described in "Methods".



d) Carnitine acyltransferase$_I$ (CAT$_I$)

The assayed employed is based on that of Saggerson (1982).

i) Incubation time

Figure 2.5 shows that the rate of reaction is linear up to at least 3 min. Subsequently an incubation time of 2 min. was adopted as routine.

ii) Enzyme concentration

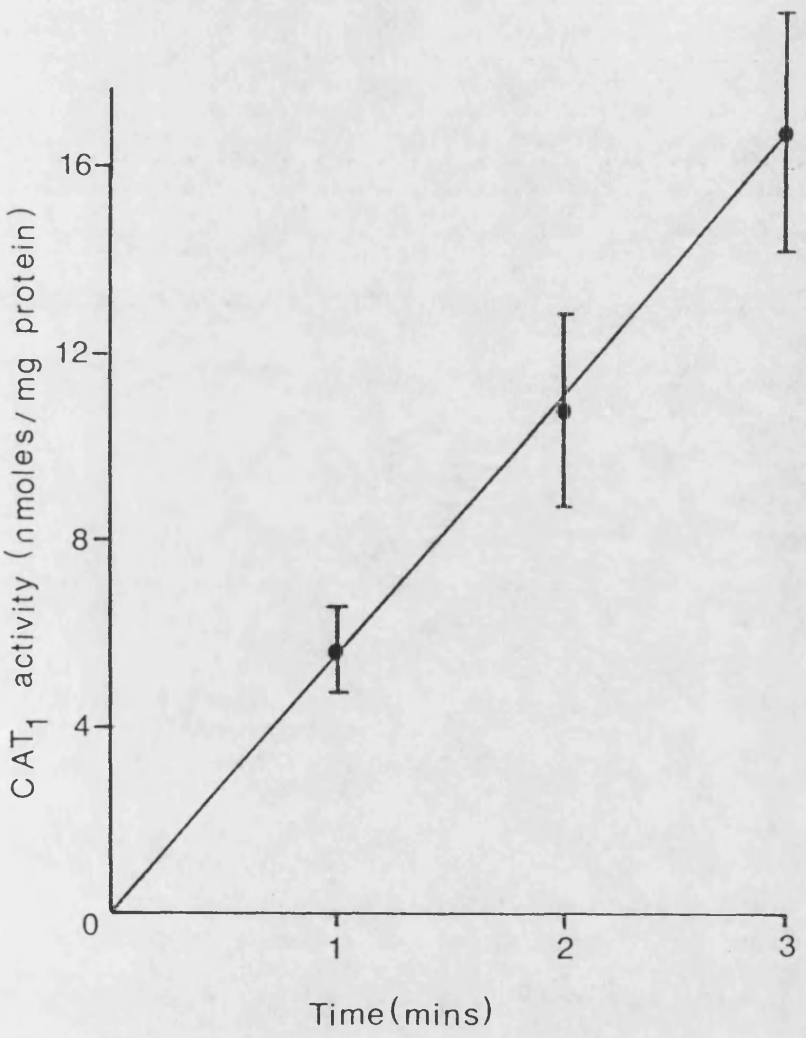
Heathers (1985) found that the assay was linear up to at least 40µg protein per assay. In the experiments described here, ≈25µg protein was added per assay.

Figure 2.5

Effect of incubation time on CAT_I activity

Number of observations at each point = 4

Values are means $\pm$ S.E.



RESULTS

3.1 GPAT activity in subcellular fractions

In adipose tissue, GPAT activity exists in both mitochondrial and microsomal fractions (Saggerson et al., 1979). The two activities have distinct properties, most noteably they show different sensitivity to sulphydryl reagents such as N-ethylmaleimide (NEM): in rat adipose tissue, NEM inhibited GPAT activity in the microsomal, but not mitochondrial fraction (Saggerson et al., 1980).

Subcellular fractions were prepared from perfused hearts as described in 'Methods'. Table 3.1 shows GPAT activity in the various fractions. The highest specific activity was found in the microsomal 200 000g pellet where the activity was approximately 10-fold higher than that of the crude homogenate.

The microsomal pellet was extracted at a protein concentration of 1 - 1.5mg/ml (above 1.7mg/ml the assay was not linear with respect to enzyme concentration (Figure 3.1)).

Heathers et al (1985) used NEM in homogenates of rat heart to distinguish between microsomal and mitochondrial activity. Samples of homogenate, mitochondrial or microsomal fractions were incubated $\pm$ 15mM NEM for 5 min. at 30^{o}C as described in 'Methods'. GPAT activity was measured immediately after the incubation and the results are shown in Table 3.2. It is clear that inhibition of GPAT activity

Table 3.1

GPAT activity in subcellular fractions

Fraction	GPAT activity (nmoles/min/mg)
Crude homogenate	.57 ± .08 (6)
2000g supernatant	.41 ± .09 (6)
10 000g supernatant	.72 ± .14 (4)
10 000g pellet	.56 ± .06 (6)
200 000g pellet	5.27 ± .40 (5)

Hearts were perfused for 20 min. before homogenisation in STE buffer. GPAT activity was measured in various subcellular fractions as described in "Methods".

Results are means ± S.E., number of observations shown in parentheses.

Figure 3.1 Effect of protein concentration on microsomal GPAT activity

Each point represents the mean of 2 observations

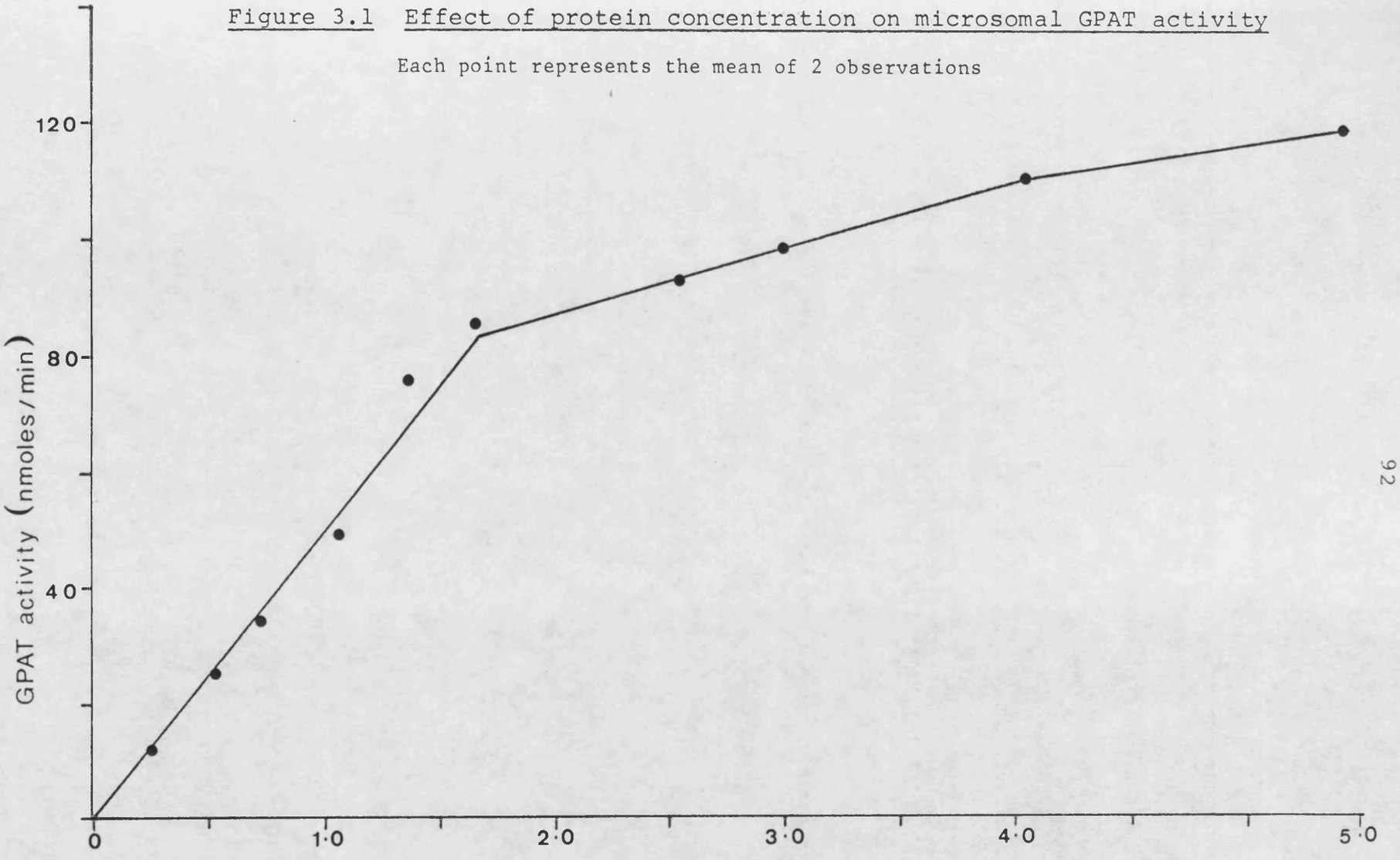


Table 3.2

Effect of N-ethylmaleimide on mitochondrial and microsomal GPAT activity

Fraction	GPAT activity (nmoles/min/mg) -NEM	+NEM
Crude homogenate	.58 ± .07 (4)	.38 ± .05 (4) *
Mitochondrial	.69 ± .05 (5)	.64 ± .05 (5)
Microsomal	5.69 ± .47 (4)	1.15 ± .23 (4) **

Hearts were perfused for 20 min. and subcellular fractions prepared as described in "Methods". Extracts were incubated ± 15mM N-ethylmaleimide for 5 min. at 30°C before measurement of GPAT activity.

Results are means ± S.E., number of observations shown in parentheses. Statistical significance (v activity in absence of NEM), ** $p < .001$; * $p < .005$.

occurs in both homogenate and microsomal fraction (35% and 80% inhibition, respectively) but not in the mitochondrial fraction.

3.2 Adrenergic effects on enzyme activity

a) Effect of perfusion with adrenaline on heart performance and the activation of TGL and GPAT

Hearts were perfused normally for 20 min. before exposure to 10^{-6}M adrenaline for 1 min. as described in 'Methods'. From Table 3.3 it can be seen that adrenaline increases both heart rate and developed tension, by 33% and 55% respectively. TGL activity is increased by 50% and GPAT activity (in crude homogenate) is depressed by 35%.

Activity of GPAT in the mitochondrial fraction is statistically unchanged by adrenaline (Table 3.3). However, microsomal GPAT activity is depressed by 16% - a statistically significant amount.

b) Effect of homogenization in NaF on GPAT activity

Isolation of the microsomal pellet takes approximately 1 h after removal of the heart from the perfusion apparatus. During this time, in adrenaline treated hearts, a partial reactivation of GPAT occurs. Thus in homogenate a 35% inhibition is seen whereas in the microsomal fraction only a 16% inhibition remains. To determine whether this reactivation was due to endogenous phosphatase activity, both control and adrenaline - perfused hearts were

Table 3.3

Effect of perfusion with 10^{-6}M adrenaline on heart performance and the activities of TGL and GPAT

	Control	Adrenaline
Heart rate (beats/min)	227 ± 17 (10)	302 ± 15 (6)**
Developed tension (g/heart)	4.9 ± .9 (12)	7.6 ± 1.2 (4)**
TGL activity } nmoles/min/mg	.10 ± .01 (6)	.15 ± .02 (6)**
GPAT activity } nmoles/min/mg		
Homogenate	.58 ± .06 (6)	.36 ± .03 (6)**
Mitochondrial	.67 ± .05 (3)	.73 ± .18 (3)
Microsomal	5.16 ± .53 (7)	4.33 ± .44 (4)*

Hearts were perfused for 20 min. without or with exposure to 10^{-6}M adrenaline for 1 min. Hearts were homogenised in STE buffer and enzyme activities measured as described in "Methods".

Statistical significance:

** $p < .001$; * $p < .025$

homogenized in buffer containing 50mM NaF (a phosphatase inhibitor).

Table 3.4 shows that in the presence of NaF, adrenaline produces a 36% inhibition of GPAT activity in the microsomal fraction. This value is similar to that for adrenaline - induced inhibition of GPAT in crude homogenate (35%) and indicates that inhibition of phosphatases allows the GPAT inhibition to be preserved during the isolation of the microsomal fraction.

c) Effect of incubation of heart extracts with cAMP, ATP and cAMP - dependent protein kinase on GPAT activity

Figure 3.2 shows that incubation of homogenate from control hearts with cAMP, ATP and cAMP-dependent protein kinase (cAMP-PrK) results in a time-dependent inactivation of GPAT with a significant inhibition from 10 min. onwards. The activity of GPAT incubated with buffer alone did not change over 30 min. This agrees with results of Heathers et al (1985) who found that inhibition of GPAT under these conditions was restricted to the microsomal (NEM-sensitive) fraction.

In microsomal pellet incubation with buffer alone, GPAT activity decreases over 30 min. (Figure 3.3). Incubating with cAMP, ATP and cAMP-PrK produces a statistically significantly greater inhibition at 15 min. and 30 min.

Table 3.4

Effect of homogenate NaF on measured GPAT activity

		GPAT activity (nmoles/min/mg)	
	NaF	Homogenate	Microsomal
Control	-	.62 ± .04 (6)[1]	5.16 ± .53 (7)[5]
	+	.64 ± .05 (4)[2]	4.24 ± .30 (4)[6]
10^{-6}M Adrenaline	-	.43 ± .04 (7)[3]	4.33 ± .38 (3)[7]
	+	.45 ± .04 (5)[4]	2.77 ± .23 (4)[8]

Hearts were perfused for 20 min. without or with exposure to 10^{-6}M adrenaline for 1 min. Hearts were homogenised in STE buffer in absence or presence of 50mM NaF. GPAT activity was measured in homogenate or microsomal pellet, as described in "Methods". When included, NaF was present throughout extraction of one microsomal fraction. Results are means ± S.E., number of observations shown in parentheses. Statistical significance: 1v3, 2v4, $p < .001$; 5v6 $p < .01$; 5v7 $p < .05$; 7v8 $p < .001$; 6v8 $p < .001$; other differences not significant.

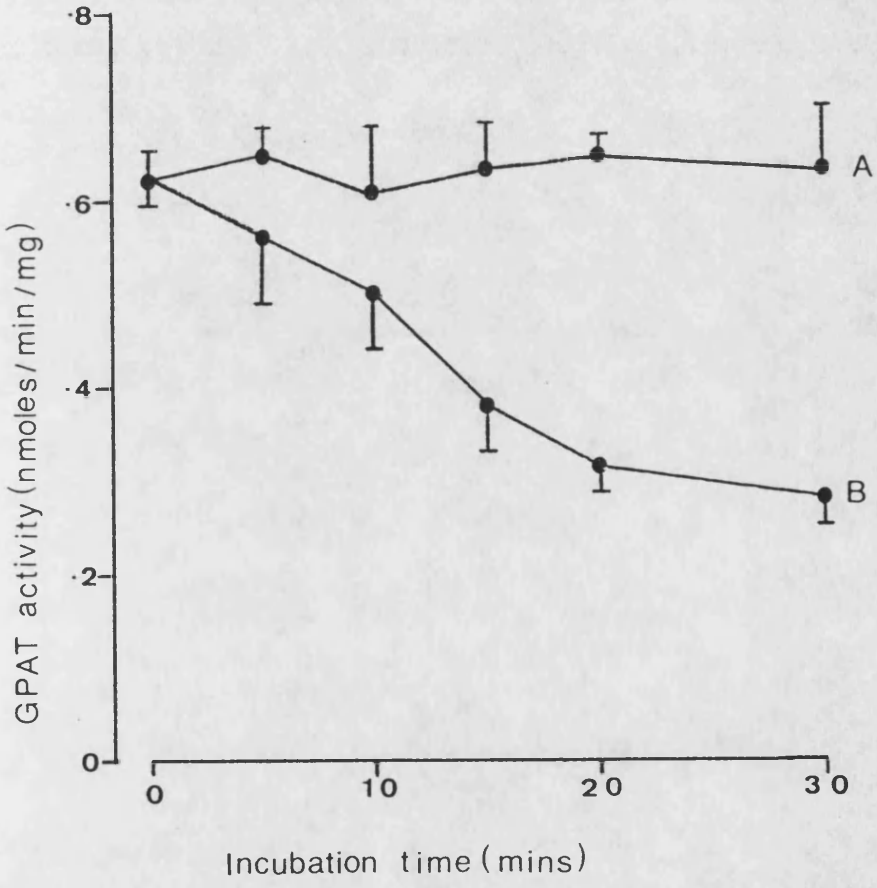
Figure 3.2

GPAT activity in heart homogenate incubated with cAMP, ATP and cAMP-PrK

Number of observations at each value = 3

Values are means ± S.E

Statistical significance: 15 min, $p < .01$; 20 min, 30 min, $p < .001$



Hearts were perfused for 20 min. before incubation of homogenate with A-buffer only, or B-cAMP, ATP and cAMP-PrK (see "Methods") for 15 min. at 30°C. Samples were taken at various time points during the incubation and assayed for GPAT activity.

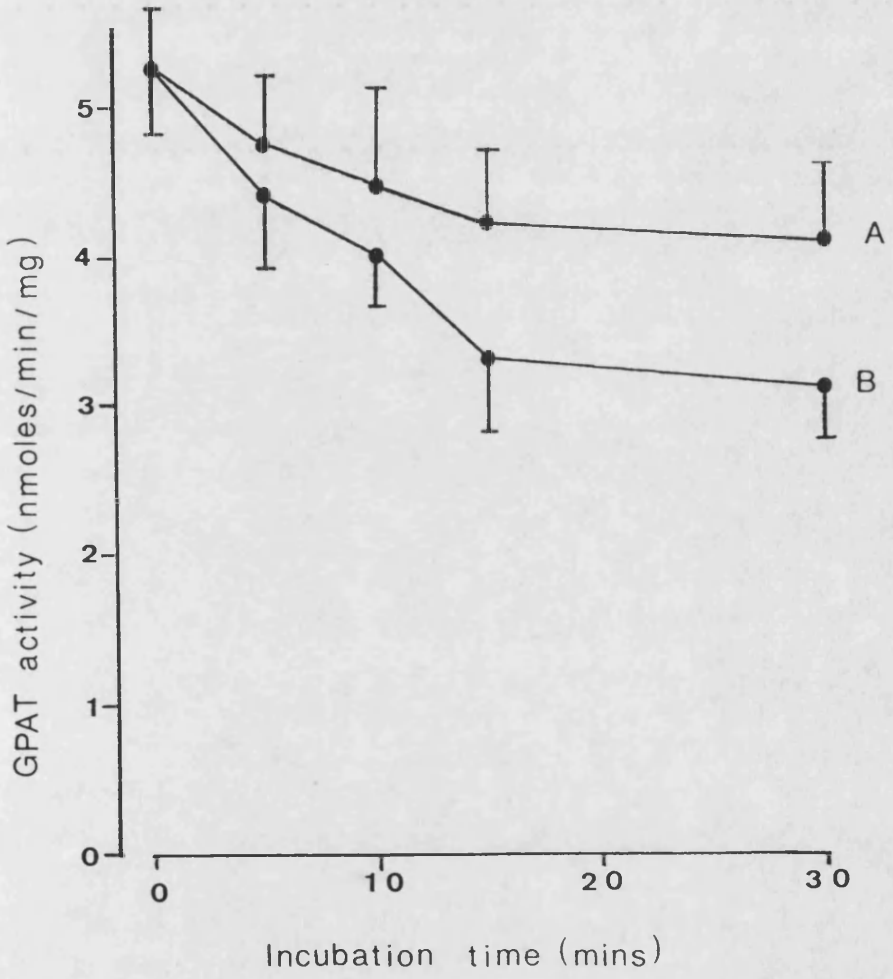
Figure 3.3

GPAT activity in microsomal fraction incubated with cAMP, ATP and cAMP-PrK

Number of observations at each value = 4

Values are means ± S.E.

A statistically significant difference in activity occurs at 15 min. ($p < .05$) and 30 min. ($p < .025$)



Hearts were perfused for 20 min. before isolation of the microsomal fraction and incubation with A-buffer only, or B-cAMP, ATP and cAMP-PrK (see "Methods") for 15 min. at 30°C. Samples were taken at several time points during the incubation and assayed for GPAT activity.

d) Effect of incubation under dephosphorylation conditions on the activities of TGL and GPAT

This method relies on activation of endogenous phosphatases by Mg^{2+} (Severson, 1977). Extracts from control and adrenaline-perfused hearts were incubated at 30°C for 30 min. under dephosphorylation conditions as described in 'Methods'.

Table 3.5 shows that in control hearts, TGL activity is decreased by 26% following incubation with dephosphorylation conditions. In adrenaline-treated compared with control hearts TGL is initially activated. Incubation with buffer alone produces a decrease in activity, but incubation with dephosphorylation conditions produces a greater inactivation.

In control hearts, GPAT activity is not significantly altered on incubation with either buffer alone or under dephosphorylation conditions (Table 3.6). In adrenaline-treated hearts, GPAT activity is depressed initially. Incubating with buffer alone produces a partial reactivation but incubating with dephosphorylation conditions produce complete reactivation.

3.3 Phorbol Esters

Heathers et al (1985) found that perfusion of hearts with β-agonists led to changes in both TGL and GPAT activities, but that only GPAT activity responded to α_1-agonists.

Table 3.5

Effect of incubation under dephosphorylation conditions on TGL activity from control and adrenaline-perfused hearts.

	TGL activity (nmoles/min/mg)	
	Control	Adrenaline
Initial activity	.094 ± .01 (6)[1]	.13 ± .02 (4)[4]
30 min. incubation: buffer only	.095 ± .02 (7)[2]	.094 ± .02 (4)[5]
dephosphorylation conditions	.070 ± .01 (6)[3]	.070 ± .01 (4)[6]

Hearts were perfused for 20 min without or with exposure to 10^{-6}M adrenaline before homogenization in STE buffer. 2000g supernatant was incubated with buffer alone or under dephosphorylation conditions as described in "Methods" for 30 min. at 30°C before assay of TGL. Results are expressed as means ± S.E., number of observations shown in parentheses. Statistical significance: 1v3, 2v3, $p < .01$; 4v6, $p < .025$

Table 3.6

Effect of incubation under dephosphorylation conditions on GPAT activity in homogenates of control and adrenaline - perfused hearts

	GPAT activity (nmoles/min/mg)	
	Control	Adrenaline
Initial activity	.59 ± .05 (6)	.45 ± .03 (5)[1]
30 min. incubation: buffer only	.58 ± .15 (4)	.54 ± .07 (5)[2]
dephosphorylation conditions	.65 ± .19 (5)	.74 ± .13 (4)[3]

Homogenates from control and 10^{-6}M adrenaline-treated hearts were incubated with buffer alone or under dephosphorylation conditions for 30 min. at 30°C as described in Methods, before assay of GPAT activity. Results are means ± S.E., number of observations shown in parentheses. Statistical significance: 1v2, 2v3, $p < .025$; 1v3, $p < .0025$

Activation of α_1-adrenergic receptors causes formation of two 2nd messengers, IP_3 and DAG. IP_3 causes an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) DAG binds to and activates protein kinase C (PKC), which also requires phospholipid and Ca^{2+} for maximum activation (review: Berridge, 1987).

Tumour-promoting phorbol esters, such as 12-O-tetradecanoyl phorbol 13-acetate (TPA), are potent activators of PKC, mimicking the action of DAG. To determine whether PKC was the mediator of the α_1-induced fall in GPAT activity, hearts were perfused with 10^{-7}M TPA for 5 min. - this is known to produce activation of PKC (Yuan et al., 1987).

a) Effect of perfusion with TPA on heart performance.

Hearts were perfused normally for 15 min. before switching to perfusate containing 10^{-7}M TPA for 5 min. Table 3.7 shows that at the end of this perfusion time, both heart rate and developed tension are significantly reduced, by 17% and 50% respectively. Perfusion pressure is increased (by 150%) indicating coronary vasoconstriction. These observations are similar to those reported by Yuan et al (1987).

b) Effect of perfusion with TPA on activities of TGL and GPAT

Hearts were perfused with 10^{-7}M TPA for 5 min. before removal and homogenization in STEF buffer.

Table 3.7

Effect of perfusion with 10^{-7}M TPA on heart performance

	Control	TPA
Heart rate (beats/min)	275 ± 37 (9)	229 ± 26 (9)*
Developed tension (g/heart)	3.8 ± 0.6 (7)	1.94 ± .49 (7)**
Perfusion pressure (mmHg)	38.1 ± 5.9 (8)	96.3 ± 16.9 (8)**

Hearts were perfused normally for 15 min. before switching to perfusate containing 10^{-7}M TPA for 5 min. Results are means ± S.E., number of observations shown in parentheses. Statistical significance: ** $p < .001$, * $p < .01$ v control

TGL activity does not change in response to TPA (Table 3.8). This is consistent with the previous observation that α_1-agonists do not affect TGL activity. Table 3.8 shows that whilst GPAT activity in mitochondria is unchanged, it is depressed in both crude homogenate and microsomal fraction - by 37.5% and 28%, respectively. Since the inhibition persists through isolation of the microsomal fraction, this suggests that a stable change in GPAT activity has occurred.

c) Effect on GPAT activity of incubation of extracts of TPA-treated hearts under dephosphorylation conditions

Hearts were perfused with 10^{-7}M TPA and homogenized in absence of NaF. Extracts were then incubated under dephosphorylation conditions as described in 'Methods' to determine whether the TPA-induced inhibition of GPAT activity could be reversed. Table 3.9 shows that incubation with buffer alone produces a partial reactivation of GPAT but that full reactivation occurred when the extracts were incubated under dephosphorylation conditions.

d) Effect of incubation with TPA on the activities of TGL and GPAT

Crude homogenate or supernatant was incubated at 30°C for 15 min. with 10^{-7}M TPA and the additions shown in Table 3.10. These conditions are such as to favour activation of protein kinase C (Castagna et al, 1982; see

Table 3.8

Effect of perfusion with 10^{-7}M TPA on the activities of TGL and GPAT

	Enzyme activity (nmoles/min/mg)	
	Control	TPA
TGL	.10 ± .01 (5)	.105 ± .02 (5)
GPAT:		
Homogenate	.64 ± .05 (4)	.40 ± .05 (5)*
Mitochondrial fraction	.67 ± .05 (3)	.70 ± .04 (3)
microsomal fraction	4.17 ± .26 (4)	3.02 ± .21 (4)*

Hearts were perfused normally for 15 min. before perfusion with 10^{-7}M TPA for 5 min. Enzyme activity was measured in the various subcellular fractions as described in "Methods". Results are means ± S.E., number of observations shown in parentheses. Statistical significance v control: * $p < .001$, other differences not significant.

Table 3.9

Effect on GPAT activity of incubation of homogenates from TPA-treated hearts under dephosphorylation conditions

	GPAT activity (nmoles/min/mg)
Initial activity	.34 ± .05 (3)[1]
30 min incubation: buffer only	.44 ± .07 (5)[2]
dephosphorylation conditions	.59 ± .07 (6)[3]

Hearts were perfused normally for 15 min. without or with perfusion with 10^{-7}M TPA for 5 min. Homogenates were incubated with buffer alone or under dephosphorylation conditions (as described in "Methods") for 30 min. at 30°C before measurement of GPAT activity. Statistical significance: 1v2, NS; 2v3; $p < .01$; 1v3, $p < .005$

Table 3.10

Effect of 10^{-7}M TPA incubation on heart homogenate enzyme activities

	Enzyme activity (nmoles/min/mg)	
	TGL	GPAT
Initial activity	.09 ± .01 (3)	.60 ± .05 (6)
15 min. incubation with buffer only	.09 ± .02 (6)	.60 ± .06 (3)[1]
ATP		.59 ± .05 (4)
ATP, PS, Ca^{2+}		.54 ± .04 (6)
ATP, TPA		.44 ± .08 (6)[2]
ATP, PS, Ca^{2+}, TPA	.09 ± .01 (6)	.33 ± .05 (6)[3]

Hearts were perfused normally for 20 min. before incubation of homogenate (GPAT) or 2000g supernatant (TGL) under the conditions given for 15 min. at 30°C (as described in "Methods"). Concentrations used were: 5mM ATP, 20µg/ml phosphatidylserine (PS), 100µM Ca^{2+}, 10^{-7}M TPA. Results are means ± S.E., number of observations shown in parentheses. Statistical significance: 1v2, $p < .005$; 1v3, $p < .001$; 2v3, $p < .025$

'Methods' for details).

Table 3.10 shows that TGL activity is unaffected on incubation with TPA, but that GPAT activity is depressed by 27%. This is consistent with the observation that α_1-agonists have no effect on TGL activity but inhibit GPAT (Heathers et al 1985). Addition of cofactors of PKC - Ca^{2+} and phosphatidylserine (PS) - caused a further inhibition of GPAT, strongly suggesting that PKC activation can inhibit GPAT activity.

e) Effect of perfusion with TPA and adrenaline

i) Heart performance

Perfusion of hearts with either adrenaline or TPA produces different effects on heart function (Table 3.11): perfusion with 10^{-6}M adrenaline for 1 min. increases both heart rate (not shown) and developed tension whereas perfusion with 10^{-7}M TPA for 5 min. has the opposite effect. Perfusion with TPA and adrenaline together (see 'Methods') has the same effect as perfusion with TPA alone, suggesting that TPA inhibits the heart's response to adrenaline.

ii) GPAT

GPAT activity was measured in the microsomal fraction (see 'Methods'). Perfusion of hearts with either adrenaline or TPA produced a decrease in GPAT activity (Table 3.12) - by 35% and 30%, respectively. Perfusion with adrenaline and TPA simultaneously produced approximately the same decrease (35%) in GPAT activity as perfusion with either agent alone.

Table 3.11

Effect of perfusion with TPA and adrenaline on developed tension

	Developed tension (g/heart)
Control	$4.9 \pm .97$ (12)[1]
Adrenaline	7.6 ± 1.2 (4)[2]
TPA	$1.9 \pm .50$ (4)[3]
TPA and adrenaline	$2.0 \pm .40$ (3)[4]

Hearts were perfused with 10^{-7}M TPA and/or 10^{-6}M adrenaline as described on "Methods". Results are means $\pm$ S.E., number of observations shown in parentheses. Statistical significance: all differences $p < .001$, except 3v4, NS.

Table 3.12

Effect of perfusion with TPA and adrenaline on microsomal GPAT activity

	GPAT activity (nmoles/min/mg)
Control	$4.27 \pm .20$ (3)[1]
Adrenaline	$2.77 \pm .23$ (4)[2]
TPA	$3.0 \pm .13$ (4)[3]
TPA and adrenaline	$2.72 \pm .24$ (3)[4]

Hearts were perfused with 10^{-7}M TPA and/or 10^{-6}M adrenaline as described in "Methods". Hearts were homogenised in buffer containing 50mM NaF and GPAT activity measured in a microsomal fraction. Results are means $\pm$ S.E., number of observations shown in parentheses. Statistical significance: 1v2, $p < .001$; 1v3, $p < .001$; 1v4 $p < .0025$. Other differences not significant.

iii) Phosphorylase

Activation of glycogen phosphorylase 'a' by both α_1 and β-adrenergic agonists is well documented (review, Hayes, 1986). Here, perfusion of hearts with 10^{-6}M adrenaline increased phosphorylase activity by 100% (Table 3.13).

Perfusion of hearts with TPA also led to a stable increase in phosphorylase activity (by 40%; table 3.13). This has not been reported previously. Perfusion of hearts with adrenaline and TPA together also gave a 40% increase in phosphorylase activity - the same result as perfusion with TPA alone. This suggests that at the concentrations used the TPA effect predominates over the adrenaline response.

3.4 Calcium and calcium antagonists

The internal calcium concentration ($[Ca^{2+}]_i$) of the myoplasm determines the level of activation of contractile proteins (review - Hiraoki and Vogel, 1987) which, in turn, controls the force of developed tension. Changes in $[Ca^{2+}]_i$ can also alter the activity of a number of other proteins and enzymes. In particular, glycogen phosphorylase activity is increased (by Ca^{2+}-induced activation of phosphorylase kinase) and that of glycogen synthase decreased (review - Hayes, 1986). To determine whether the activities of enzymes involved in triglyceride metabolism

Table 3.13

Effect of perfusion with TPA and adrenaline on phosphorylase activity

	Phosphorylase activity (-AMP/+AMP) ratio
Control	$.15 \pm .01$ (3)[1]
Adrenaline	$.32 \pm .04$ (3)[2]
TPA	$.21 \pm .01$ (4)[3]
TPA and adrenaline	$.21 \pm .01$ (3)[4]

Hearts were perfused with 10^{-7}M TPA and/or 10^{-6}M adrenaline as described in "Methods". Hearts were homogenised in buffer containing 50mM NaF and phosphorylase activity determined in a 10 000g supernatant (after gel filtration - see "Methods"). Results are means $\pm$ S.E., number of observations shown in parentheses. Statistical significance: 1v2, 1v3, 1v4, $p < .01$; 2v3, $p < .0025$; 2v4, $p < .05$

are also influenced by Ca^{2+}, the heart was perfused with various concentrations of external Ca^{2+} ($[Ca^{2+}]_o$) and also with Ca^{2+} uptake antagonists.

(Hearts were homogenised in STE buffer and GPAT activity measured in crude homogenate in the results presented in this section).

a) Effect on heart performance

i) $[Ca^{2+}]_o$

Figure 3.4 shows that as the $[Ca^{2+}]_o$ of the perfusate is raised from 0.6 to 4.8mM, developed tension increases (heart rate does not change - results not shown). This result is consistent with the proposal that as $[Ca^{2+}]_o$ is increased, $[Ca^{2+}]_i$ is also increased. As discussed above, this increases the activity of contractile proteins.

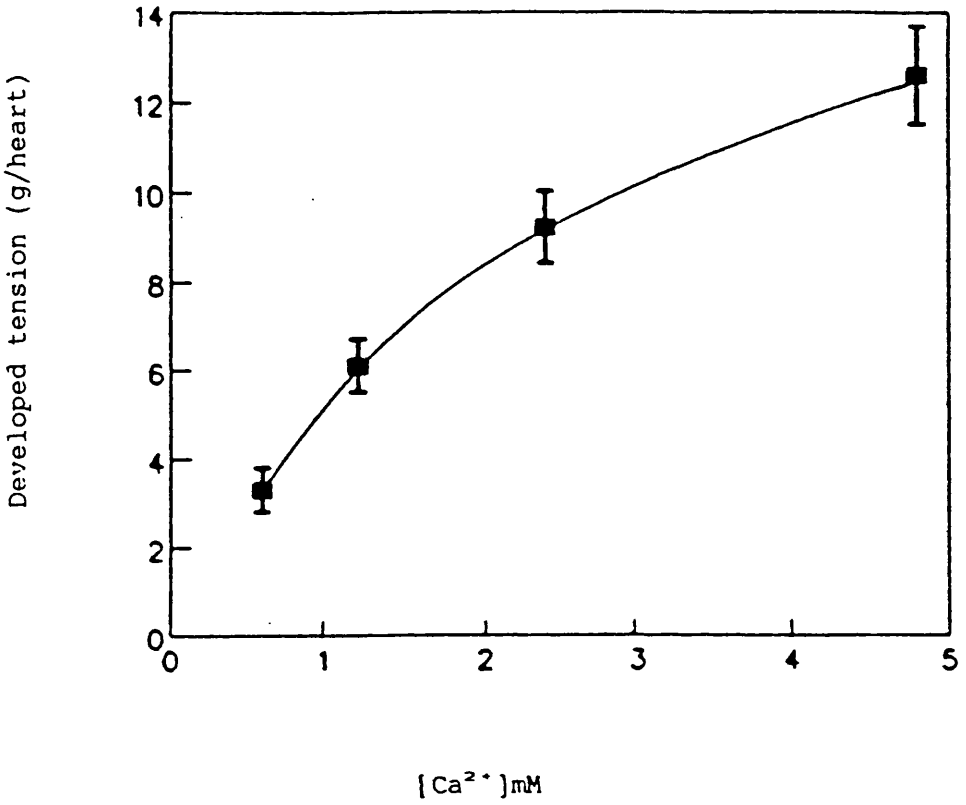
ii) Ca^{2+} antagonists

Ca^{2+} antagonists bind to the slow calcium channel of the sarcolemma and inhibit Ca^{2+} entry. The structures of diltiazem, nifedipine and verapamil and their properties are given in the introduction.

The effect of perfusion with Ca^{2+} antagonists on inotropy and chronotropy in the isolated rat heart is shown in Table 3.14; hearts were perfused normally for 15 min. before switching to perfusate plus antagonist for 5 min. At the concentrations shown, diltiazem and verapamil produced

Figure 3.4

Effect of changes in perfusate [Ca^{2+}] on developed tension



Hearts were perfused (resting tension 2g) with a control [Ca^{2+}] of 1.2mM for 15 min before the perfusate was switched to [Ca^{2+}] of 0.6, 1.2, 2.4 or 4.8mM for 5 min. Results are means ± S.E, number of observations for each value =6. Statistical significance v control (1.2mM Ca^{2+}) : 0.6mM Ca^{2+} $p<.02$; 2.4mM Ca^{2+} $p<.02$; 4.8mM Ca^{2+} $p<.01$.

TABLE 3.14 The effect of Ca^{2+} antagonists on heart rate and developed tension

Antagonist	Developed tension (g/heart)	Heart rate (beats/min)
Control	3.24 ± .38 (18)	208 ± 23 (18)
Diltiazem, 10^{-6}M	2.05 ± .14 (7) **	162 ± 19 (7) **
Nifedipine, 10^{-7}M	2.16 ± .21 (6) **	169 ± 17 (6) *
Verapamil, 10^{-7}M	1.72 ± .19 (5) **	175 ± 10 (5) *

Hearts (resting tension 2g) were perfused normally for 15 min before the given concentrations of antagonist were introduced for 5 min. Values are expressed as means ± S.E, number of observations (hearts) shown in parentheses. Statistical significance v control; * $p<.01$, ** $p<.001$.

an approximate 50% decrease in developed tension whereas nifedipine had a lesser effect. Heart rate was reduced by ≈20% in each case. Higher concentrations of nifedipine (10^{-6}M) and verapamil (10^{-6}M) reduced developed tension to zero and produced very low heart rates (results not shown).

b) Effect on TGL and GPAT activities

i) $[Ca^{2+}]_o$

Hearts were perfused with $[Ca^{2+}]_o$ of 0.6 to 4.8mM. TGL activity is reduced by 40% at $[Ca^{2+}]$ of 0.6mM (Table 3.15) compared to the control value at 1.2mM. At concentrations above the control value, no further increase in TGL activity occurs. GPAT activity is strongly inhibited (>50%) at $[Ca^{2+}]_o$ above 1.2mM; at 0.6mM Ca^{2+}, a slight but significant activation occurs.

ii) Ca^{2+} antagonists

All three Ca^{2+} antagonists inhibited TGL activity (Table 3.16); diltiazem and nifedipine produced an approximate 25% decrease in activity whereas verapamil causes a 45% decrease. Since both developed tension and heart rate are reduced in presence of these antagonists, the reduced TGL activity may reflect a reduced energy demand by the heart. Diltiazem and verapamil produce a significant increase in GPAT activity (Table 3.16)whereas the slight activation produced by nifedipine does not reach statistical significance.

TABLE 3.15 The effect on enzyme activity of changes in perfusate [Ca^{2+}]

	TGL activity (nmoles/min/mg)	GPAT activity (nmoles/min/mg)
0.6mM Ca^{2+}	.06 ± .01 (4) ***	.68 ± .05 (4) *
1.2mM Ca^{2+}	.10 ± .02 (5)	.57 ± .07 (6)
2.4mM Ca^{2+}	.09 ± .01 (6)	.29 ± .02 (6) **
4.8mM Ca^{2+}	.10 ± .02 (6)	.24 ± .06 (6) ***

Hearts were perfused normally for 15 min before being switched to perfusate containing the given [Ca^{2+}] for 5 min. At this time hearts were removed and enzyme activities determined. Values are expressed as means ± S.E., number of observations shown in parentheses. Statistical significance v 1.2mM Ca^{2+} * $p<.05$, ** $p<.005$, *** $p<.001$.

TABLE 3.16 The effect on enzyme activity of Ca^{2+} antagonists

	TGL activity	GPAT activity
	(nmoles/min/mg)	
Control	.10 ± .01 (6)	.58 ± .06 (6)
Diltiazem, 10^{-6}M	.08 ± .01 (6) ***	.67 ± .06 (6) *
Nifedipine, 10^{-7}M	.08 ± .01 (6) ***	.64 ± .07 (6)
Verapamil, 10^{-7}M	.06 ± .01 (5) ****	.78 ± .11 (6) **

Hearts were perfused normally for 15 min before the Ca^{2+} antagonist was introduced into the perfusate at the given concentration for 5 min. At this time hearts were removed and enzyme activities determined. Values are expressed as means ± S.E., number of observations shown in parentheses. Statistical significance v relevant control: * $p<.05$, ** $p<.01$, *** $p<.005$, **** $p<.001$.

c) Effect of preperfusion with diltiazem on enzyme activity during ischaemia and reperfusion

$[Ca^{2+}]_i$ is known to increase during ischaemia and reperfusion (Jennings et al., 1985). To determine whether blockage of Ca^{2+} entry during ischaemia and reperfusion would alter enzyme activity, diltiazem was introduced into the perfusate 5 min. before induction of ischaemia and remained present throughout ischaemia and reperfusion.

Induction of regional ischaemia for 10 min. leads to a 50% increase in TGL activity (Table 3.17) which is restored to pre-ischaemic values on reperfusion. This agrees with previous work from this laboratory (Heathers and Brunt, 1985). Although perfusion with diltiazem decreased TGL activity initially (Table 3.17), the same degree of activation of TGL occurs during iscnaemia of diltiazem-treated hearts as occurs in control hearts. On reperfusion, TGL activity is again restored to pre-ischaemic values. Thus diltiazem does not affect the ischaemia-induced increase in TGL activity.

Compared with the non-ischaemic area, GPAT activity is reduced by 30% in the ischaemic area of control hearts (Table 3.18). On reperfusion, GPAT activity is further reduced to approximately 50% of the pre-ischaemic value. Perfusion with diltiazem does not alter the ischaemia-induced fall in GPAT activity. However, on reperfusion of hearts perfused with diltiazem, GPAT activity returned to pre-ischaemic values, i.e. blockage of Ca^{2+} entry on

TABLE 3.17

The effect on TGL activity of perfusion with diltiazem during ischaemia and reperfusion

	TGL activity (nmoles/min/mg)			
	Control hearts		Diltiazem-treated hearts	
	NI	I	NI	I
10 min ischaemia	.11 ± .01 (6)	.16 ± .001 (6)*	.05 ± .01(7)	.08 ± .01(7)*
10 min ischaemia + 5 min reperfusion	.10 ± .01(6)	.10 ± .01(6)	.06 ± .01(6)	.06±.01(8)

Control and diltiazem-perfused hearts were subjected to 10 min regional ischaemia with or without 5 min reperfusion. Tissue samples were taken from the non-ischaemic (NI) and ischaemic (I) areas and TGL activity determined as detailed in 'Methods and Materials'. Values are expressed as means ± S.E., number of observations shown in parentheses. Statistical significance for ischaemic v non-ischaemic areas: *p<.001.

TABLE **3.18** The effect on GPAT activity of perfusion with diltiazem during ischaemia and reperfusion

	GPAT activity (nmoles/min/mg)			
	Control hearts		Diltiazem-treated hearts	
	NI	I	NI	I
10 min ischaemia	.64 ± .10 (6)	.45 ± .05 (6)*	.74 ± .04 (7)	.51 ± .06 (7)*
10 min ischaemia + 5 min reperfusion	.67 ± .13 (6)	.34 ± .11 (6)*	.64 ± .06 (8)	.67 ± .09 (8)

Control and diltiazem perfused hearts were subjected to 10 min regional ischaemia with or without 5 min reperfusion. Tissue samples were taken from the non-ischaemic (NI) and ischaemic (I) areas and GPAT activity determined as described in 'methods and materials'. Values are expressed as means ± S.E., number of observations shown in parentheses. Statistical significance for ischaemic v non-ischaemic areas:*$p<.001$.

reperfusion prevented the reperfusion-induced decrease in GPAT activity.

d) Effect of Ca^{2+} in the GPAT assay buffer

The above results suggest that increases in $[Ca^{2+}]_i$ can inhibit GPAT activity. To determine whether this was a direct effect of Ca^{2+} or whether another mediator, such as a Ca^{2+}-dependent protein kinase., was involved, the GPAT assay was performed using buffers of known $[Ca^{2+}]$ (see "Methods" for preparation of these buffers).

Table 3.19 shows that $[Ca^{2+}]$ of 10 - 100 μM has no effect on GPAT activity. The free $[Ca^{2+}]_i$ of the myoplasm is normally in the range 0.1 to 10 μM (Marban et al.,1980). Therefore, it appears that Ca^{2+} does not directly inhibit GPAT but must be working via another mediator, such as a Ca^{2+}-dependent protein kinase.

3.5 The Diabetic Heart

Rats were made diabetic by a single injection of streptozotocin and hearts isolated 2 days later, as described in "Methods". This model of diabetes has been reported to produce elevated blood levels of glucose and ketone bodies (Jenkins, 1986).

Table 3.19

Effect on GPAT activity of Ca^{2+} in the assay buffer

$[Ca^{2+}]$ (μM)	GPAT activity (nmoles/min/mg)
10	.64 ± .06 (5)
50	.61 ± .09 (5)
100	.61 ± .06 (5)

GPAT activity was measured in homogenates of perfused hearts. Assay buffer of known $[Ca^{2+}]$ were prepared as described in "Methods". Results are means ± S.E., number of observations shown in parentheses. There are no statistically significant differences.

GPAT activity in this section was measured in the microsomal pellet. For ischaemic studies, hearts were made globally ischaemic as it was difficult to obtain enough material from the regionally ischaemic model to measure GPAT activity in a microsomal fraction. Hearts were homogenized in buffer containing 50mM NaF.

Diabetes causes increased circulating levels of free fatty acids which are responsible for the increased triglyceride content of the diabetic heart (Feuvray et al 1979). However, in these experiments hearts were perfused with normal concentrations of glucose in order to make a direct comparison with results obtained from hearts of normal rats.

a) Heart performance

After 20 min. of normal perfusion, heart function is depressed in this model of diabetes; heart rate by 30% and developed tension by 25% (Table 3.20).

b) Enzyme activity during ischaemia and reperfusion

i) TGL

Following 20 min. normal perfusion, basal TGL activity is depressed by 60% in diabetic hearts compared with normals (Table 3.21). Induction of 10 min. ischaemia causes a 50% activation of TGL in normal hearts, whereas a 100% activation occurs in hearts from diabetic rats.

Table 3.20

Effect of diabetes on heart function

	Heart rate (beats/min)	Developed tension (g/heart)
Control	286 ± 30 (9)	5.2 ± 1.0 (9)
Diabetic	203 ± 19 (7)**	3.9 ± .50 (7)*

Hearts from normal and diabetic rats were perfused for 30 min. with a resting tension of 2g as described in "Methods". Results are means ± S.E., number of observations shown in parentheses. Statistical significance v control: * $p < .005$, ** $p < .001$

Table 3.21

Effect of ischaemia on TGL activity in hearts from normal and diabetic rats

	TGL activity (nmoles/min/mg)	
	Normal	Diabetic
Control	.11 ± .01 (6)[1]	.04 ± .007 (4)[3]
10 min ischaemia	.16 ± .01 (6)[2]	.07 ± .02 (4)[4]

Hearts from normal and diabetic rats were perfused normally for 10 min. before induction of 10 min ischaemia, as described in "Methods"; TGL activity was determined in the 2000g supernatant. Results are means ± S.E., number of observations shown in parentheses. Statistical significance: 1v2, 1v3, 2v4, 3v4, $p < .001$

ii) CAT_I

The diabetic heart contains elevated levels of long chain acyl carnitine (LCAC) (Feuvray et al 1979) and inhibition of LCAC formation has been reported to improve performance of diabetic hearts (Rosen et al 1986). We therefore studied the activity of the enzyme responsible for LCAC formation, namely, CAT_I. Table 3.22 shows that CAT_I activity is unchanged in perfused hearts from diabetic rats. Also, neither induction of 10 min. ischaemia nor 1 min. reperfusion caused any significant changes in CAT_I activity in hearts from either normal or diabetic rats.

iii) GPAT

Basal microsomal GPAT activity is slightly, but significantly, depressed in perfused hearts from diabetic rats (Table 3.23). Induction of 10 min. global ischaemia produces a similar decrease in GPAT activity in both normal and diabetic hearts - 35% and 27% respectively. 1 min. reperfusion led to a further depression in GPAT activity in normal hearts. However, on reperfusion of diabetic hearts no further significant decrease in GPAT activity occurred.

iv) Phosphorylase

Basal phosphorylase activity is elevated by 70% in this model of diabetes (Table 3.24). Induction of ischaemia in normal hearts causes a 33% activation of phosphorylase, whereas a smaller activation occurs during ischaemia of diabetic hearts (17%). On reperfusion of normal hearts, a

Table 3.22

Effect of ischaemia and reperfusion on CAT_I activity in hearts from normal and diabetic rats

	CAT_I activity (nmoles/min/mg)	
	Normal	Diabetic
Control	$5.37 \pm .61$ (4)	$5.67 \pm .83$ (5)
10 min ischaemia	$5.58 \pm .47$ (4)	$5.41 \pm .41$ (3)
10 min ischaemia & 1 min reperfusion	$5.41 \pm .29$ (3)	$5.25 \pm .92$ (4)

Hearts from normal and diabetic rats were perfused for 10 min. before induction of 10 min ischaemia without or with 1 min reperfusion, as described in "Methods". CAT_I activity was measured in the mitochondrial fraction (10 000g pellet). Results are means $\pm$ S.E., number of observations shown in parentheses. There are no statistically significant differences (i.e. $p > .05$) between any of the groups.

Table 3.23

Effect of ischaemia and reperfusion on microsomal GPAT activity in hearts from normal and diabetic rats

	GPAT activity (nmoles/min/mg)	
	Normal	Diabetic
Control	$4.27 \pm .20$ (3)[1]	$3.17 \pm .27$ (3)[4]
10 min ischaemia	$2.81 \pm .39$ (5)[2]	$2.38 \pm .33$ (3)[5]
10 min ischaemia and 1 min reperfusion	$2.18 \pm .31$ (3)[3]	$2.09 \pm .41$ (3)[6]

Hearts from normal and diabetic rats were perfused normally for 10 min. before induction of 10 min. ischaemia with or without 1 min. reperfusion, as described in "Methods". Control hearts were perfused normally for 20 min. GPAT activity was measured in the microsomal fraction (200 000g pellet). Results are means $\pm$ S.E., number of observations shown in parentheses. Statistical significance: 1v2, $p < .001$; 1v3, $p < .001$; 2v3, $p < .05$; 4v5, $p < .05$; 4v6, $p < .025$; 1v4, $p < .005$; other differences not significant.

Table 3.24

Effect of ischaemia and reperfusion on phosphorylase activity in hearts from normal and diabetic rats

	Phosphorylase activity (-AMP/+AMP) ratio	
	Normal	Diabetic
Control	.15 ± .01 (3)	.24 ± .025 (3)[4]
10 min ischaemia	.20 ± .03 (3)[2]	.28 ± .01 (3)[5]
10 min ischaemia and 1 min reperfusion	.26 ± .04 (5)[3]	.30 ± .06 (3)[6]

Hearts from normal and diabetic rats were perfused for 10 min before induction of 10 min. ischaemia without or with 1 min. reperfusion. Control hearts were perfused for 20 min. Phosphorylase activity was determined in the 10 000g supernatant following gel filtration, as described in "Methods". Results are means ± S.E., number of observations shown in parentheses. Statistical significance: 1v2, $p < .025$; 1v3, $p < .0025$; 2v3, $p < .05$; 4v5, $p < .05$; 1v4, $p < .0025$; 2v5, $p < .01$ Other differences not significant.

further significant activation of phosphorylase occurs beyond that seen during ischaemia. However, on reperfusion of diabetic hearts there was no further increase in phosphorylase activity.

c) Effect of adrenaline on enzyme activity

Hearts from both normal and diabetic rats were perfused for 20 min. before exposure to 10^{-6}M adrenaline for 1 min. as described in "Methods".

i) TGL

10^{-6}M adrenaline produces a 50% rise in TGL activity in normal hearts (Table 3.25). Basal TGL activity is depressed in diabetic hearts, but perfusion with adrenaline induces a 100% rise in activity.

ii) CAT_I

The activity of this enzyme is not under the direct control of adrenergic hormones i.e. does not undergo phosphorylation. This is shown here in Table 3.26, where there is no change in CAT_I activity following perfusion with 10^{-6}M adrenaline in hearts from either normal or diabetic rats.

Table 3.25

Effect of perfusion with adrenaline on TGL activity in hearts from normal and diabetic hearts

	TGL activity (nmoles/min/mg)	
	Normal	Diabetic
Control	$.10 \pm .01$ (6)[1]	$.04 \pm .005$ (7)[3]
10^{-6}M adrenaline	$.15 \pm .02$ (6)[2]	$.08 \pm .015$ (6)[4]

Hearts from normal and diabetic rats were perfused for 20 min. without (control) or with 10^{-6}M adrenaline for 1 min. TGL activity was determined in a 2000g supernatant, as described in "Methods". Results are means $\pm$ S.E., number of observations shown in parentheses. Statistical significance: 1v2, 1v3, 3v4, 2v4, $p < .001$

Table 3.26

Effect of perfusion with adrenaline on CAT_I activity in hearts from normal and diabetic rats

	CAT_I activity (nmoles/min/mg)	
	Normal	Diabetic
Control	5.37 ± .61 (4)	5.84 ± .42 (3)
10^{-6}M adrenaline	5.57 ± .83 (5)	5.09 ± .59 (3)

Hearts from normal and diabetic rats were perfused for 20 min. without (control) or with exposure to 10^{-6}M adrenaline for 1 min. as described in "Methods". CAT_I activity was measured in the mitochondrial fraction (10 000g pellet). Results are means ± S.E., number of observations shown in parentheses. There are no statistically significant differences between any of the groups.

iii) GPAT

In normal hearts, exposure to 10^{-6}M adrenaline caused a 35% decrease in GPAT activity measured in the microsomal fraction. In diabetic hearts, the depressed basal GPAT activity is further inhibited by adrenaline (A 35% decrease in activity), (Table 3.27).

iv) Phosphorylase

Activation (phosphorylase by adrenaline is well known. Here, perfusion with 10^{-6}M adrenaline caused a 100% activation of phosphorylase in normal hearts (Table 3.28). Basal phosphorylase activity is increased in diabetes but adrenaline produces a further 100% increase in activity (Table 3.28).

Table 3.27

Effect of perfusion with adrenaline on microsomal GPAT activity in hearts from normal and diabetic rats.

	GPAT activity (nmoles/min/mg)	
	Normal	Diabetic
Control	$4.47 \pm .20$ (3)[1]	$3.32 \pm .28$ (3)[3]
10^{-6}M adrenaline	$2.77 \pm .23$ (4)[2]	$2.16 \pm .35$ (3)[4]

Hearts from normal and diabetic rats were perfused for 20 min. without (control) or with exposure to 10^{-6}M adrenaline for 1 min. GPAT activity was determined in the microsomal fraction (200 000g pellet). Results are means $\pm$ S.E., number of observations shown in parentheses. Statistical significance: 1v2, $p < .001$; 1v3, $p < .005$; 3v4, $p < .025$; 2v4, $p < .05$

Table 3.28

Effect of perfusion with adrenaline on phosphorylase activity in hearts from normal and diabetic rats

	Phosphorylase activity(-AMP/+AMP) ratio	
	Normal	Diabetic
Control	$.15 \pm .006$ (3)[1]	$.24 \pm .014$ (3)[3]
10^{-6}M adrenaline	$.32 \pm .035$ (4)[2]	$.51 \pm .066$ (3)[4]

Hearts from normal and diabetic rats were perfused for 20 min. without (control) or with exposure to 10^{-6}M adrenaline for 1 min. Phosphorylase activity was determined in a 10 000g supernatant after gel filtration, as described in "Methods". Results are means $\pm$ S.E., number of observations shown in parentheses. Statistical significance: 1v3, $p < .0025$; 1v2, $p < .01$; 3v4, $p < .01$; 2v4, $p < .05$.

DISCUSSION

4.1 Adrenergic effects on the activities of triglyceride lipase and glycerol 3-phosphate acyltransferase

a) Triglyceride lipase

In the heart, hydrolysis of endogenous triglyceride is under acute hormonal control (Severson, 1979): perfusing the isolated rat heart with catecholamines increases glycerol output and reduces the level of myocardial triglyceride (Crass et al., 1975). Triglyceride lipase (TGL) catalyses the initial, rate limiting step in triglyceride hydrolysis (Stam et al., 1986) however, the nature of TGL has been controversial: a residual form of lipoprotein lipase (Hulsmann et al., 1982; Palmer and Kane, 1983) versus a distinct neutral lipase (Goldberg and Khoo, 1985; Ramirez et al., 1985). Most recent evidence is in favour of the latter (Holm et al., 1988; Small et al., 1989) and the assay employed here to determine TGL activity was designed to distinguish between it and LPL (Al-Muhtaseb, 1983).

In adipose tissue, the existence of a neutral triglyceride lipase has been known for some time and it has been termed hormone-sensitive lipase since it is phosphorylated and activated in response to catecholamines (Belfrage et al., 1977). Recently, the gene for hormone sensitive lipase has been cloned from rat adipocytes (Holm et al., 1988); the predicted amino acid sequence showed no homology with that of lipoprotein lipase, despite similar functions of the lipases, and RNA blot analysis revealed that the gene for hormone sensitive lipase was also present in rat heart.

Thus the existence of neutral TGL in heart muscle is now well established and it is also agreed that its activity can be stimulated by catecholamines (Heathers et al., 1985; Table 3.3, this thesis, Severson et al., 1980). However, whether a direct phosphorylation of the enzyme by cAMP-PrK occurs (as in adipose tissue) has again been controversial. TGL activity was found to increase on incubation of rat (Heathers et al., 1985) and mouse (Goldberg and Khoo, 1985) heart extracts with cAMP, ATP and cAMP-PrK. A similar result was obtained by Schoonderwoerd et al., (1987) using rat heart, but these workers suggested that the increase in TGL activity was not due to phosphorylation of the enzyme. Instead, they suggested that TGL activity was increased as a result of removal of inhibitory fatty acids from the catalytic site of the enzyme by re-esterification: cAMP-dependent phosphorylation of phosphorylase leads to a stimulation of glycogenolysis thereby increasing the supply of glycerol 3-phosphate. This latter would then react with fatty acids to re-form triglyceride However, our observation that myocardial GPAT activity is decreased on incubation of heart extracts with cAMP-PrK (Heathers et al., 1985; Figures 3.2 and 3.3, this thesis) does not support this hypothesis - a depressed GPAT activity would inhibit re-esterification of fatty acids. Also, Palmer et al., (1987) observed the increase in TGL activity with cAMP-PrK even when their assay system was depleted of glycogen. Recently, evidence for direct phosphorylation of myocardial TGL was provided by Small et al., (1989): incubation of rat

heart extracts with cAMP-PrK and (γ^{32}P)ATP caused phosphorylation of an 84K polypeptide which could be precipitated by antiserum raised against purified hormone-sensitive lipase from adipose tissue. The experiment was repeated using isolated myocytes to exclude the possibility that TGL was derived from intramuscular adipocytes or other cell types.

Thus most recent evidence is strongly in favour of a distinct TGL in heart muscle which is phosphorylated and activated by cAMP-PrK upon catecholamine-induced stimulation of the heart.

β-adrenergic agonists only are effective in stimulating TGL, perfusion of isolated rat hearts with α_1-agonists had no effect on TGL activity (Heathers et al., 1985). TGL activity is inhibited by high concentrations of free fatty acid and fatty acyl CoA (Severson, 1979; Al-Muhtaseb, 1982) but whether this inhibition would overcome the activation produced by phosphorylation is not known and requires further investigation.

b) Glycerol 3-phosphate acyltransferase

Most work outside this laboratory has focussed on glycerol 3-phosphate acyltransferase (GPAT) of adipose tissue (Nimmo, 1984; Rider and Saggerson, 1983). There is no conclusive evidence as to whether the adipose tissue enzyme is under acute hormonal control: Rider and Saggerson (1983) observed a stable decrease in microsomal, but not mitochondrial, GPAT activity in response to incubation of

rat adipocytes with noradrenaline; however, no change in activity was found when untreated adipocytes were incubated with cAMP-PrK. This is in contrast to results obtained by Nimmo (1980; 1981) who found that GPAT activity was depressed on incubation of a microsomal fraction from rat adipocytes with cAMP-PrK. Initially, this was suggested to be due to cAMP-dependent phosphorylation of GPAT, but in a later paper (Nimmo and Nimmo, 1984) the band phosphorylated with (γ^{32}P)ATP (upon SDS-PAGE of adipocyte microsomes incubated with (γ^{32}P)ATP and the catalytic subunit of cAMP-PrK) was found to differ in molecular weight by 500-1000 from that thought to be GPAT (54K). No alternative mechanism for the cAMP-induced inhibition of GPAT was proposed. A recent paper by Walsh et al., (1989) found adipocyte microsomal GPAT was inhibited on incubation with ATP alone, and that no further inhibition occurred on adding cAMP-PrK, suggesting that a cAMP-independent phosphorylation was involved.

Previous work from this laboratory found a decrease in myocardial GPAT activity in crude homogenate following perfusion of isolated rat hearts with adrenaline (Heathers et al., 1985). The present study confirms this observation - GPAT activity was depressed by 38% in response to adrenaline (Table 3.3) and further shows that, upon isolation of subcellular fractions, mitochondrial GPAT activity was unchanged but that of the microsomal fraction depressed by 16% (which was statistically significant). Since GPAT activity in homogenate was inhibited by nearly 40%, this

suggested that a re-activation of GPAT occurred during isolation of the microsomal fraction (which took approximately 1 hr. following removal of the heart from the perfusion apparatus). If GPAT is indeed phosphorylated by adrenaline, it is possible that the reactivation was due to the action of endogenous phosphoprotein phosphatases. To determine whether this was the case, following perfusion (without or with adrenaline) the heart was homogenised in buffer containing 50mM NaF (a phosphatase inhibitor). There was no further inhibition of GPAT in crude homogenate (Table 3.4) but activity in the microsomal fraction was depressed by 36%, suggesting that phosphatase action was indeed responsible for the reactivation of GPAT. This is further evidence that GPAT is phosphorylated and inactivated in response to adrenaline.

Rider and Saggerson (1983) observed a large decrease in microsomal GPAT activity on incubation of rat adipocytes with noradrenaline, NaF had no effect on this decrease, however, if albumin was included in the homogenization buffer, the noradrenaline-induced inhibition was abolished. No explanation was given for the effect of albumin. The effect of albumin on myocardial GPAT inactivation has not been studied. However, it may be that different mechanisms operate for the control of GPAT in heart and adipose tissue: Specific GPAT activity is approximately ten times higher in adipose tissue where the concentrations of triglyceride and fatty acid metabolites would be much higher than in heart

muscle. It is possible that such metabolites have an important role in regulating adipose tissue GPAT activity whereas in heart the main control mechanism is by phosphorylation. The susceptibility of the dephosphorylated and phosphorylated forms of the enzyme to control by factors such as fatty acids and metabolites needs to be investigated.

The depression in myocardial GPAT activity (in crude homogenate) produced by adrenaline can be reversed on incubation of homogenates under dephosphorylation conditions (Heathers et al., 1985; Table 3.5). Incubation of both homogenate and microsomal fraction of untreated hearts with cAMP-PrK also produces a decrease in GPAT activity (Figures 3.2 and 3.3).

Perfusion of isolated rat hearts with both α_1 and β-adrenergic agonists can inhibit GPAT (Heathers et al., 1985). Whereas the results of this study and previous work show that the β-receptor mediated inhibition may be due to cAMP-dependent phosphorylation of GPAT, the mechanism of α_1-induced inhibition was not known. Investigation of this has been the subject of much of the work of this thesis and is discussed in Sections 4.3 and 4.4.

From the results of this section it can be concluded that when the heart is challenged by an adrenergic stimulus, endogenous triglycerides are mobilized by activation of TGL whilst at the same time wasteful recycling of fatty acids is depressed by inhibition of GPAT activity. In absence of the hormonal stimulus, the activities of these enzymes may be governed by the supply of substrates.

4.2. Ischaemia and reperfusion-induced changes in enzyme activity

The levels of free fatty acid, fatty acyl CoA and acylcarnitine rise during ischaemia (Feuvray et al., 1979). However, only after long (>1 hour) periods of ischaemia could a change in the level of triglyceride or phospholipid be observed in the ischaemic heart (van Bilsen et al., 1989). It has been suggested that these conflicting results arise from the fact that current assay methods are not sensitive enough to measure small changes in triglyceride levels (van Bilsen et al., 1989). Thus a 5% decrease in triglyceride levels (which might be disregarded as being insignificant) can provide a 5-fold increase in fatty acid levels. The increase in fatty acids and metabolites occurring during ischaemia has been associated with cell damage and implicated in the genesis of ischaemia-induced arrhythmias (Corr et al., 1984). Altered control of lipid metabolism is thus an important area of investigation for the ischaemic myocardium.

An increased release of endogenous noradrenaline occurs in the ischaemic myocardium, as discussed in more detail in the 'Introduction'. This has also been implicated in arrhythmogenesis since blockage of both α and β-adrenergic receptors is beneficial in protecting against ischaemia and reperfusion-induced arrhythmias.

Previous work has shown that, on induction of regional

ischaemia in the isolated perfused rat heart, a rise in TGL activity and a fall in GPAT activity occurred in the ischaemic area (Heathers and Brunt, 1985). This has been confirmed in the present study (Tables 3.17 and 3.18). Preperfusion of the heart with β-, but not α_1-adrenergic antagonists prevented these changes in enzyme activity suggesting an increased β-adrenergic activity during ischaemia. Other workers have provided evidence for an increase in the actual number of β-receptors in ischaemic tissue (Maisel et al., 1987). On reperfusion of the previously ischaemic area, TGL activity returned to control pre-ischaemic values, but GPAT activity was further depressed (Heathers and Brunt, 1985). This reperfusion-induced fall in GPAT activity was prevented by α_1- but not β -antagonists, suggesting that, on reperfusion, β-adrenergic activity returns to normal but an α_1- drive becomes apparent. Other workers have also observed an increase in α_1-activity on reperfusion of the myocardium - this has been described in Section 1.

The work in this study has confirmed the changes in TGL and GPAT activity occurring upon induction of regional ischaemia, and also show that similar changes occur when the globally ischaemic model is used. Using this model it was possible to obtain enough tissue to measure GPAT activity in the microsomal pellet. Phosphorylase activity in the 10 000g

supernatant of the same tissue was also measured. Control of phosphorylase activity by both α_1 and β-adrenergic mechanisms is well known (review Hayes, 1986) and it was therefore desirable to compare changes in phosphorylase activity with changes in GPAT activity. NaF was included in the homogenisation buffer to prevent the action of phosphatases on the enzymes.

After 10 min. global ischaemia, microsomal GPAT activity was found to decrease by 35%, and phosphorylase activity to increase by 35% (Tables 3.23 and 3.24). On reperfusion for 1 min., a further depression of GPAT activity and a further elevation of phosphorylase activity occurred. Both these changes in activity were very stable. Thus isolation of the microsomal pellet took approximately 1 h. following removal of the heart from the perfusion apparatus and phosphorylase activity was assayed after gel filtration of the 10 000g supernatant. Thus phosphorylation of the enzymes seems the most likely explanation for the observed changes in activity. The reperfusion-induced fall in GPAT activity can be prevented by α_1-antagonists in the regionally ischaemic model. It would be interesting to determine whether preperfusion of the heart with adrenergic antagonists prevents the ischaemia and reperfusion-induced changes in phosphorylase activity as has been found with the GPAT activity. Further work needs to be carried out to determine whether the changes in GPAT, TGL and phosphorylase activities

during ischaemia can be correlated with changes in cAMP levels and cAMP-PrK activity. If α_1-activity is indeed responsible for the reperfusion-induced changes in GPAT and phosphorylase activities, the mechanism of the changes will need to be established. α_1-Activation produces an increase in $[Ca^{2+}]_i$ and activates protein kinase C (PKC). It has been shown that an increase in $[Ca^{2+}]_i$ occurs during reperfusion of the myocardium (Shen and Jennings, 1972). The influence of such an increase on GPAT activity is discussed in Section 4.3. Whether PKC activation can alter the activities of GPAT and phosphorylase is discussed in Section 4.4. It would be of great interest to determine the changes in PKC activity occurring during ischaemia and reperfusion of the heart.

4.3. Calcium and calcium antagonists.

Stimulation of α_1-receptors causes both an increase in $[Ca^{2+}]_i$ and an activation of protein kinase C (PKC). A possible mediator of the α_1-induced fall in GPAT activity is an increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ increases during reperfusion of the myocardium (Shen and Jennings, 1972) when, as we have shown, there is a decrease in GPAT activity. This reperfusion-induced decrease in GPAT activity can be prevented by pre-perfusing the heart with α_1-antagonists (Heathers and Brunt, 1985) and evidence for such intensification of α_1-activity during reperfusion has been found elsewhere (Stewart et al., 1980; Sharma et al., 1983).

a) Effect on enzyme activity

To determine whether alterations in $[Ca^{2+}]_i$ would alter enzyme activity, hearts were perfused with various $[Ca^{2+}]_o$ (0.6 - 4.8mM) and with the Ca^{2+} antagonists diltiazem, nifedipine and verapamil. These all act by inhibiting the Ca^{2+} channel and thus reducing Ca^{2+} entry into the myocardium. Increasing $[Ca^{2+}]_o$ increased developed tension. This result suggests that $[Ca^{2+}]_i$ was increased which led to an activation of contractile proteins. All three Ca^{2+} antagonists reduced both heart rate and developed tension (Table 3.14). This well established property (review Fleckenstein, 1984) has been suggested to account, at least in part, for the anti-arrhythmic action of Ca^{2+} antagonists. Here the results obtained (Table 3.16) show that TGL activity is inhibited by both Ca^{2+} antagonists and by low $[Ca^{2+}]_o$ (0.6mM). The activity appears maximal at 1.2mM $[Ca^{2+}]_o$. The responsiveness of TGL to Ca^{2+} requires further investigation which would involve purification of myocardial TGL and accurate estimations of $[Ca^{2+}]_i$.

GPAT activity is strongly inhibited in response to high $[Ca^{2+}]_o$ whereas, compared to control values at 1.2mM $(Ca^{2+})_o$, either low $[Ca^{2+}]_o$ or Ca^{2+} antagonists produce an increase in activity.

The enzyme activities at high $[Ca^{2+}]_o$ are similar to those found on reperfusion of the isolated ischaemic rat heart when $[Ca^{2+}]_i$ is known to increase (review, Jennings et al., 1985).

b) Effect of pre-perfusion with diltiazem on enzyme activity during ischaemia and reperfusion

During reperfusion $[Ca^{2+}]_i$ is raised by Ca^{2+} influx from outside the cell. The Ca^{2+} entry mechanisms are not fully understood but may involve a stimulation of Ca^{2+} slow channels by (1) catecholamine-induced phosphorylation (2) palmitoyl carnitine accumulation (Mir and Spedding, 1986) or (3) α_1-stimulated Ca^{2+} influx (Sharmaet al., 1983). If Ca^{2+} antagonists prevent the reperfusion induced increase in $[Ca^{2+}]_i$, alterations in Ca^{2+}-sensitive enzyme activity might be expected. Inclusion of diltiazem in the perfusate prior to induction of regional ischaemia caused an inhibition of TGL activity in both non-ischaemic and ischaemic tissue (Table 3.17). This effect was similar to that seen with low $[Ca^{2+}]_o$ and suggests that the treatment was successful in limiting Ca^{2+} influx. The extent of TGL stimulation caused by ischaemia in the presence of diltiazem was identical to that seen in its absence. This suggests that such ischaemic effects are entirely β-mediated as has been argued earlier.

On reperfusion, diltiazem did not influence the return of TGL activity to pre-ischaemic values. It did not prevent the ischaemia-induced inhibition of GPAT activity (Table 3.18). However, pre-perfusion with diltiazem did prevent the reperfusion-induced fall in GPAT activity.

A similar result had been obtained with the α_1-antagonist doxazosin (Heathers and Brunt, 1985). Thus this result suggests that the α_1-induced effect was caused by Ca^{2+} entry on reperfusion which inhibited GPAT. It is unlikely that Ca^{2+} directly inhibits GPAT since the inclusion of 10-100μM Ca^{2+} in the GPAT assay buffer had no effect on activity (Table 3.19). It is possible that Ca^{2+} entry on reperfusion activates a Ca^{2+}-dependent protein kinase which via phosphorylation inhibited GPAT activity.

Thus under control conditions it is suggested that the reperfusion-induced fall in GPAT activity would delay removal of β-adrenergically generated fatty acids or their metabolites accumulating during ischaemia (Corr et al., 1984), and that this would delay the recovery of heart performance. Diltiazem prevents the reperfusion-induced fall in GPAT activity and provides another mechanism whereby Ca^{2+} antagonists protect against reperfusion-induced injury of the myocardium.

4.4. Effects of phorbol esters (protein kinase C activation)

Stimulation of the heart with α_1-adrenergic agonists results in the formation of two 2nd messengers - IP_3 which causes an increase in $[Ca^{2+}]_i$, and diacylglycerol (DAG), which activates protein kinase C (PKC) (review - Berridge, 1987) The effect of changes in $[Ca^{2+}]$ on enzyme activity has been discussed in Section 4.3. The experiments discussed in this section were designed to investigate the effects on

enzyme activity of the other half of the α_1-signalling pathway, namely PKC activation. For this reason, phorbol esters, which are potent activators of PKC (Castagna et al., 1982), were used.

Phorbol esters have a similar structure to that of DAG and activate PKC directly both in vivo and in vitro (Niedel et al., 1983). PKC requires Ca^{2+} and phospholipid (particularly phosphatidyl-serine) for activity (review, Nishizuka, 1986); phorbol esters and DAG increase the activity of PKC even at maximal Ca^{2+} concentrations. More importantly they decrease the concentration of Ca^{2+} required for half maximal activity. This is reduced to the submicromolar range found in the cytosol (Exton, 1988). A major difference between phorbol esters and DAG is that whereas DAG is present for only a short time following cell stimulation, phorbol esters are hardly degraded by the cell and so may prolong the activation of PKC. This may distort the normal sequence of events. Therefore, care must be taken in interpreting results obtained with phorbol esters (Exton, 1988).

PKC can phosphorylate a large number of proteins in vitro but it is not known whether these are also physiological substrates for the enzyme (Nishizuka, 1986). Addition of phorbol esters to perfused hearts or isolated myocytes results in phosphorylation of several proteins, but the function of these is not yet known (Katoh et al., 1981; Presti et al., 1985; Yuan and Sen, 1986). Thus, although PKC has been purified from heart muscle (Kuo et al., 1984) much work is needed to clarify the role of PKC in vivo.

a) Heart mechanics

Perfusion of isolated rat hearts with the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) has been shown to cause activation of PKC (Yuan et al., 1987).

The results given in Table 3.7 show that perfusion of isolated rat hearts for 5 min. with 10^{-7}M TPA causes a marked decrease in developed tension and also in heart rate. This agrees with results of Yuan et al., (1987) who used various phorbol esters, including an inactive analogue (i.e. did not activate PKC); the latter had no effect on heart performance indicating that the phorbol ester effects were due to PKC activation and not to some structural properties of the drugs. Addition of TPA to cultured chick myocytes also produces a reduced contractility (Leatherman et al., 1987). Thus the effects of phorbol esters on heart function are very different to those of catecholamines and it has been suggested that a transient versus persistent activation of PKC may account for these differences (Henrich and Simpson, 1988).

Exactly how PKC exerts this inhibitory effect on heart function is not known but *in vitro* PKC can phosphorylate a number of proteins involved in the contractile response in heart e.g. troponin-I and the C-protein of the thick filament (Katoh et al., 1983), and can also modulate Ca^{2+} channels in other cells (Rane and Dunlop, 1986; Vergilio et al., 1986). However, the functional significance of phosphorylation of these proteins by PKC *in vivo* (if any) is not yet understood.

b) TGL and GPAT activities

Perfusion of hearts with α_1-agonists inhibits GPAT activity but has no effect on TGL activity (Heathers et al., 1985). To determine whether PKC activation would produce similar changes, enzyme activity was measured in crude homogenate (GPAT) or 2000g supernatant (TGL) following perfusion of hearts with 10^{-7}M TPA for 5 min. Table 3.8 shows that no change in TGL activity occurred whereas GPAT activity was depressed by 38%. Thus it is possible that PKC is indeed the mediator of the α_1-induced inhibition of GPAT.

TPA has a vasoconstrictor effect on the heart (as indicated by the rise in perfusion pressure) and therefore may cause some degree of hypoxia or ischaemia. This is unlikely to be the reason for the decrease in GPAT activity since there is no concomittant change in TGL activity (which has been shown to increase during ischaemia).

Homogenates of TPA-treated hearts were then incubated under dephosphorylation conditions to determine whether the inhibition of GPAT activity could be reversed, as for adrenaline treated hearts. Table 3.9 shows that a partial restoration of activity occurred on incubation with buffer alone whereas a full reactivation was produced under dephosphorylation conditions. Therefore it appears that

phosphorylation of GPAT occurs in response to PKC activation.

Heathers et al., (1985) showed that cAMP-PrK could inhibit GPAT and activate TGL in vitro. Purified PKC is not yet available commercially, but phorbol esters can activate PKC in vitro (Niedel et al., 1983). Heart extracts were therefore incubated under conditions designed to optimize PKC activation (Castagna et al., 1982) and enzyme activities determined following the incubation (Table 3.10). No change in TGL activity occurred, but GPAT activity was depressed in homogenate incubated with 10^{-7}M TPA (and ATP). A further decrease in GPAT activity was observed on addition of phosphatidylserine and Ca^{2+} (which are cofactors of PKC) to the incubation. This latter observation strongly suggests that PKC activation is indeed responsible for the inhibition of GPAT.

The results discussed in this section have shown that GPAT activity is inhibited following either perfusion of hearts with TPA or incubation of heart extracts with TPA in vitro, whereas TGL activity is unaffected. Therefore, PKC activation can inhibit GPAT and this inhibition can be reversed under dephosphorylation conditions. Whether this involves a direct phosphorylation of GPAT by PKC can be determined only when GPAT has been further purified.

c) Effect of adrenaline and phorbol ester on the activities of microsomal GPAT and phosphorylase

The previous section showed that perfusion of hearts with TPA inhibits GPAT activity in crude homogenate. Table 3.12 indicates that this inhibition persists through isolation of the microsomal fraction (mitochondrial GPAT activity being unaffected by TPA) and is therefore a very stable effect. This can be compared with the effect of adrenaline on GPAT activity (Table 3.12). Perfusion of hearts with TPA and adrenaline together resulted in a similar inhibition of GPAT to that produced with either TPA or adrenaline alone (approximately 35% inhibition) indicating that the drugs do not act in an additive or synergistic manner.

Phosphorylase is activated by α_1-agonists and recent evidence using hepatocytes indicates that Ca^{2+} may not be the sole mediator of α_1-induced stimulation of phosphorylase (Saz et al., 1989): in hepatocytes fully preloaded with Ca^{2+}, α_1-agonists could elicit a further stimulation of phosphorylase. Perfusion of hearts with TPA in these experiments lead to a 40% activation of phosphorylase (Table 3.13) indicating that PKC activation can cause phosphorylation of phosphorylase. The Ca^{2+}-induced activation of phosphorylase kinase and hence phosphorylase is well known (review Hayes, 1986) and in our experiments it would have been of interest to use the Ca^{2+} ionophore A23187 together with phorbol ester to try and elucidate the interactions between the two 2nd messengers.

In liver, for example, phorbol esters alone have no effect on phosphorylase activity (van der Werve et al., 1985; Fain et al., 1984) but enhanced the activation produced with A23187.

The mechanism of the TPA, and hence PKC, activation of phosphorylase is not known. PKC is unlikely to activate phosphorylase kinase since, in vitro, this enzyme is not a substrate of PKC (Berridge, 1987). A 2nd possibility is that PKC activates a phosphatase inhibitor protein which would increase phosphorylase activity by preventing its dephosphorylation. Such a mechanism has been shown for cAMP-PrK (Gorris et al., 1984). Whether PKC does indeed act on such a protein requires investigation.

Comparing the effects of TPA and adrenaline on phosphorylase activity shows that the stimulation by adrenaline is greater than that of TPA (100% v 40%), (Table 3.12). Perfusion of hearts with TPA and adrenaline together resulted in a 40% activation of phosphorylase i.e. an identical result to that obtained with TPA alone. Together with the observation that adrenaline has no effect on heart function in the presence of TPA (Table 3.11) this suggests that TPA inhibits the mechanical and metabolic responses of the heart to adrenaline. In hepatocytes phorbol esters have been found to inhibit the α_1-induced stimulation of glycogenolysis (Corvera and Garcia-Sainz, 1984) and on

smooth muscle cells a desensitization of the α_1-receptor on addition of TPA has been observed (Leeb-Lundberg et al., 1985). In addition, phorbol esters may uncouple the β-receptor from adenylate cyclase in some cell types (Kelleher et al., 1984; Garte and Belman, 1980). We have shown that TPA inhibits the response of the heart to adrenaline, but whether TPA is acting on α_1- or β-receptors, or both, requires further investigation using specific agonists.

4.5. The diabetic myocardium

The diabetic heart contains elevated levels of triglyceride, free fatty acid, fatty acyl CoA and acylcarnitine (Feuvray et al., 1979; Paulson and Crass, 1982). Such metabolites have been associated with abnormal cardiac function and in the genesis of ischaemia and reperfusion-induced arrhythmias (Corr et al., 1984). Also, a higher rate of ventricular failure during ischaemia of diabetic rat hearts has been observed (Feuvray et al., 1979). Thus an understanding of the control of enzyme activities involved in endogenous lipid metabolism is an important area of investigation for the diabetic myocardium.

a) Heart mechanics

A depressed heart function has been observed in animal models of chronic (> 4 week) diabetes, this includes heart rate and contractility and also a ventricular 'stiffening' (McNeill and Tahiliani, 1986). Table 3.20 shows that in the

model of acute diabetes employed here, heart function was depressed; heart rate by 30% and developed tension by 25%. Such impaired mechanical performance has not often been observed in acute diabetes. It is possible that this is due to the higher dose of streptozotoccin used (120mg/kg) compared with other workers (60 - 100mg/kg). This would be expected to increase the severity of the diabetic state.

b) Effect of ischaemia and reperfusion on enzyme activities

During ischaemia of perfused hearts from normal rats, the activities of the rate-limiting enzymes for triglyceride hydrolysis - TGL, and synthesis - GPAT, are increased and decreased respectively. This has been discussed in Section 4.2. These changes in activity can be prevented by pre-perfusion of the heart with β-adrenergic antagonists and are likely to be due to cAMP-dependent phosphorylation of the enzymes (Heathers and Brunt, 1985). On reperfusion of the heart, TGL activity returns to pre-ischaemic values indicating removal of the β-adrenergic drive. However, GPAT activity is further depressed. This reperfusion-induced fall in GPAT activity is prevented by α_1-, but not β-, antagonists. Glycogen phosphorylase activity is also under the control of both α_1 and β-receptors (Hayes, 1986) and adrenergic activation of phosphorylase is altered during diabetes (Miller, 1983; Vadlamudi and McNeill, 1983).

CAT_I activity is not under acute adrenergic control and measurement of its activity can be used as an indicator of any general myocardial changes due to factors other than by these hormones.

Therefore, the activities of TGL, GPAT and phosphorylase (as hormonally reactive systems) and CAT_I (as a non-reactive system) were measured to determine whether the response of the enzymes to ischaemia and reperfusion was altered during diabetes.

i) $\underline{CAT_I}$

No persistent effects of hormones on extracted CAT_I activity in any tissue have been reported. However, activity in brown adipose tissue was found to decrease in diabetes (Jamal and Saggerson, 1988) and that of liver to increase (Harano et al., 1972). The liver activity also showed a decreased sensitivity to the physiological regulatory molecule of CAT_I - malonyl CoA (Cook et al., 1984). Table 3.22 shows that CAT_I activity was unchanged in perfused hearts from diabetic rats, also, there was no change in activity during ischaemia and reperfusion in hearts from both normal and diabetic animals.

ii) TGL

In diabetes, TGL activity has been reported to decrease in perfused rat hearts (Rosen et al., 1981; Stam et al., 1984) but to increase in myocytes from diabetic rats

(Ramirez and Severson, 1986). Table 3.21 shows that TGL activity was depressed in this model of diabetes. This is difficult to explain in view of the increased lipolysis (triglyceride breakdown and glycerol release) observed in diabetic hearts perfused with glucose as the only substrate (Paulson and Crass, 1982: Rosen et al., 1981). Ischaemia produced a greater stimulation of TGL activity in diabetic, compared with normal hearts (Table 3.21). This suggests that although there is a lower level of basal TGL activity present in diabetes a greater adrenergic drive is produced by this intervention thereby increasing cAMP levels.

iii) GPAT

In diabetic hearts, a 22% reduction in GPAT activity was observed (Table 3.23). This could be caused by increased $[Ca^{2+}]_i$ (Heyliger et al., 1987) which in turn would activate Ca^{2+}-dependent protein kinases to cause inhibition of GPAT (see Sections 4.3 and 4.4). In normal hearts, ischaemia results in a decrease in GPAT activity which has been argued to involve cAMP-dependent phosphorylation of the enzyme (Heathers and Brunt, 1985); on reperfusion, GPAT activity is significantly depressed below the ischaemic value (Table 3.23). Perfusion with both α_1 and Ca^{2+} antagonists prevents the reperfusion induced fall in GPAT activity (Heathers and Brunt, 1985; Table 3.18, this thesis). This suggests that a Ca^{2+}-

dependent process is involved. Since the change in activity is a stable one, inhibition of GPAT could be due to phosphorylation by Ca^{2+}-dependent kinase. Although ischaemia causes inhibition of GPAT in diabetic hearts, on reperfusion, no further inhibition occurs (Table 3.23). This is in contrast to normal hearts and suggests that, in diabetic hearts, Ca^{2+} entry into the myocardium is impaired during reperfusion. Such a decreased Ca^{2+}uptake has been reported, (Tani and Neely, 1988) and diabetic hearts are also reported to have a reduced Ca^{2+} uptake on increases in external [Ca^{2+}] (Gøtzche, 1983).

. iv) Phosphorylase

Basal phosphorylase activity in diabetic hearts has been reported to be unchanged (Vadlamudi and McNeill, 1983) or slightly increased(Ingebretsen et al., 1981). Table 3.24 shows that a highly significant increase in activity occurred in this model of diabetes. This result, as argued for GPAT, may be explained by an increased $[Ca^{2+}]_i$. This would activate phosphorylase kinase (PhK) which, in turn, would phosphorylate and thereby activate phosphorylase.

During ischaemia of normal hearts, an activation of phosphorylase was observed (Table 3.24) and on reperfusion, a further increase in activity occurred. These changes are the mirror image of the changes in GPAT activity during ischaemia and reperfusion and it is likely that similar

mechanisms are involved i.e. cAMP-dependent and Ca^{2+}-dependent phosphorylation. On ischaemia of diabetic hearts, phosphorylase activity was elevated but not to the same extent as in normal hearts (Table 3.24). On reperfusion no further activation of phosphorylase occurred. Again this reflects the results with GPAT and would be consistent with a depressed Ca^{2+} entry (and hence reduced activity of Ca^{2+}-dependent kinases) on reperfusion of the diabetic myocardium. Such a reduced Ca^{2+} uptake may have a protective effect since Ca^{2+} entry on reperfusion has been implicated as a key factor in the genesis of reperfusion-induced arrhythmias (Jennings et al., 1985).

c) Effects of adrenaline on enzyme activity

The activities of both TGL and GPAT are under β-adrenergic control as has already been discussed (Section 4.1); TGL activity is increased and GPAT activity decreased on perfusion of the isolated rat heart with β-agonists (Heathers et al., 1985). In addition, GPAT activity is inhibited by α_1-agonists.

Glycogen phosphorylase, also activated by both α_1- and β-receptors, has been reported to have a hypersensitive response to β-agonists in acutely diabetic rat hearts (Miller, 1983; Vadlamudi and McNeill, 1983). CAT_I activity is not under direct hormonal control. Therefore, we investigated the response to adrenaline of TGL, GPAT and phosphorylase (as reactive systems) and CAT_I (as a non-reactive system) in isolated perfused hearts from diabetic rats.

Table 3.26 shows that adrenaline produces no change in CAT_I activity in hearts from either normal or diabetic rats. CAT_I is inhibited by malonyl CoA and changes in the concentration of this regulatory molecule produced either by adrenaline or by induction of diabetes may alter the activity of CAT_I in vivo, this requires further investigation.

Basal TGL activity is reduced in diabetes (discussed in Section 4.5b). However, perfusion with adrenaline causes a greater stimulation of activity in diabetic hearts (Table 3.25). The increase in cAMP levels and protein kinase activity produced by adrenaline is similar in both normal and diabetic hearts (Miller, 1983). However, it has been suggested that diabetes disrupts the compartmentalization of cAMP within cells (Vadlamudi and McNeill, 1983) since, in diabetic hearts, phosphorylase was activated by PGE, a compound that increases cAMP levels without activating phosphorylase in normal hearts. Whether this can explain the increased TGL activity produced by adrenaline in diabetic hearts remains to be investigated.

Perfusion with 10^{-6}M adrenaline produced a similar decrease in GPAT activity (35%) in hearts from both normal and diabetic rats (Table 3.27) although basal GPAT activity was depressed in diabetes. Basal phosphorylase activity was increased by 63% in diabetes (Table 3.28) but the increase in activity produced by adrenaline was of the same order (two fold) in both normal and diabetic hearts. The changes in basal activities of GPAT and phosphorylase can be explained by an increased $[Ca^{2+}]_i$ of the myocardium (Section 4.3).

To determine whether the response of GPAT and phosphorylase to adrenaline is really altered in diabetes, it would be necessary to carry out a timecourse of the effect of adrenaline on enzyme activity.

As already mentioned, other workers have observed a hypersensitive response of phosphorylase to β-agonists in diabetic hearts, although the increases in cAMP levels, protein kinase activity and also phosphorylase kinase activity were identical to those seen in normal hearts (Miller, 1983, Vadlamudi and McNeill, 1983). Increases in $[Ca^{2+}]_i$ or disrupted compartmentalization of cyclic nucleotides may explain the altered response of phosphorylase, but this requires further investigation.

It would also be of interest to determine the response of the enzymes to pure α_1-agonists in diabetic hearts. An altered Ca^{2+} handling by the diabetic myocardium has been reported - by both the sarcoplasmic reticulum (Lopaschuk et al., 1983) and sarcolemma (Tani and Neely, 1988) but whether the increased $[Ca^{2+}]_i$ observed with α_1- agonists in normal hearts is altered in diabetes is not known. Also, there are no reports on the activity of PKC in diabetic hearts. Thus much work is required to provide an understanding of metabolic response to adrenergic stimulation in diabetic hearts.

Further work

1. Purification of GPAT and direct demonstration of phosphorylation using cAMP-dependent protein kinase.

2. Investigation of the interaction of the phosphorylated and dephosphorylated forms of TGL and GPAT with free fatty acids and fatty acyl CoA.

3. Investigation of the mechanism of protein kinase C-induced inactivation of GPAT and activation of phosphorylase.

4. Measurement of glycogen synthase activity during ischaemia and reperfusion.

5. Investigation of the effects of preperfusion with β-, α_1- and Ca^{2+} antagonists on phosphorylase and glycogen synthase activity during ischaemia and reperfusion.

6. Investigation of the effect of TPA on adrenergic receptors using specific α_1 and β-agonists, possibly using cardiac myocytes.

7. Perfusion of diabetic hearts under 'diabetic'conditions i.e. elevated levels of fatty acids and ketone bodies, to determine the effect on enzyme activity.

8. Effect of perfusion on diabetic hearts with α_1- and β-agonists on GPAT, TGL and phosphorylase activities; time-course and dose dependence of the effect of such agonists on enzyme activity.

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